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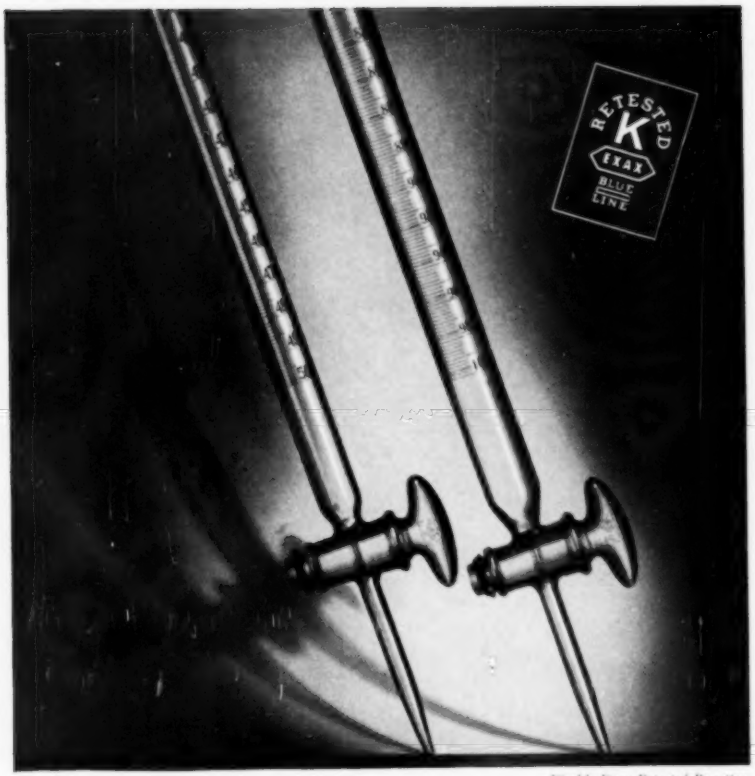

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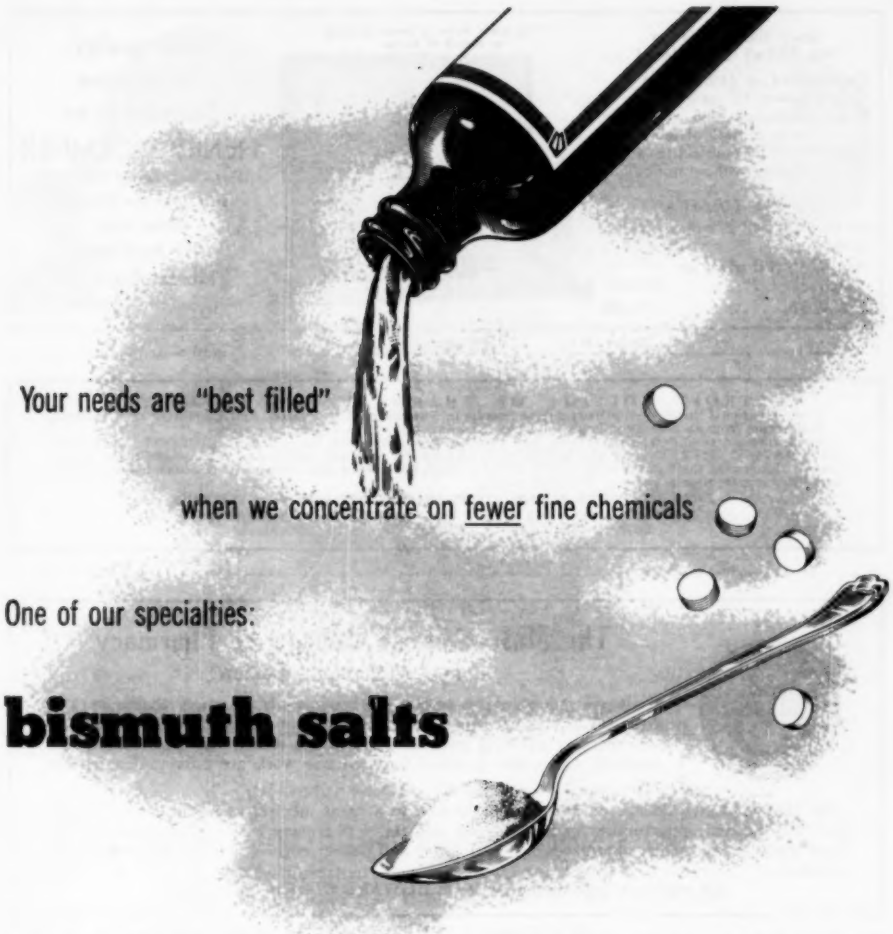

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The Relation of Chemical Structure to Analgesic Action*,†

By NATHAN B. EDDY‡

MUCH speculation and an enormous amount of work by many investigators have been devoted to the relation of chemical structure and analgesic action, stimulated anew by the discoveries of meperidine and methadone. It is not possible to review here all of the speculations but it should be worth while to consider the directions which the work has taken and to attempt to assess the progress which has been made.

MORPHINE AND ITS MODIFICATIONS

No small part of the work and speculation, naturally, has been concerned with morphine and has been designed (*a*) to elucidate its structure, (*b*) to produce and compare modifications of its molecule, and (*c*) to build up new compounds with significant analgesic action from moieties of that molecule.

(*a*) The structure of morphine is now clear (Fig. 1) except for the relative position of the nitrogen ring and it is reasonably certain that one point of closure of this ring is at carbon 9. Whether or not the other point of attachment is at carbon 13 should be established by work now going forward in our laboratories.

(*b*) The morphine molecule can and has been attacked at many points (1–3) and the changes involved are briefly summarized in Table I.

(*c*) As to moieties of the morphine molecule, one can see in the morphine framework a number of simpler structures (Fig. 2) and it is easy to imagine that the whole molecule is built around any one of them. Each of the moieties by itself is relatively inert and completely devoid of analgesic action. Nevertheless, with everyone of them as the starting point new compounds have been synthesized which exhibit some degree of analgesic effect.

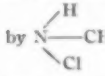
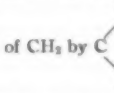
MORPHINE SYNTHESIS

If the nitrogen ring in morphine is closed at carbon 13 (Fig. 1), it and the adjacent aliphatic ring constitute a partially hydrogenated isoquinoline nucleus. Starting from isoquinoline the German chemist, Grewe (4), has synthesized a morphinan structure, which has many of the characteristics of the morphine molecule, and 3-hydroxy-N-methylmorphinan, which is even more morphine-like (Fig. 3). The latter has all of the features of morphine except the oxygen bridge, the alcoholic hydroxyl and one double bond in the aliphatic ring; in other words, it is desomorphine minus the oxygen bridge. Morphinan has been shown to produce some analgesic effect and 3-hydroxy-N-methylmorphinan is at least as effective as morphine in this respect and more prolonged in its action. Compared to desomorphine 3-hydroxy-N-methylmorphinan is a less effective but longer-acting analgesic agent. Apparently again the absence of the oxygen bridge has decreased analgesic action.

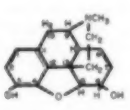
Very recently Grewe (5) has reported the synthesis of tetrahydrodesoxycodeine and has proved that it is identical chemically with tetrahydrodesoxyco-

* Received January 28, 1950, from the Federal Security Agency, National Institutes of Health, Bethesda, Md.
† Read before the graduate students in chemistry and pharmacology, Yale University School of Medicine, New Haven, Conn., December 13, 1949.
‡ Medical Officer, Experimental Biology and Medicine Institute.

TABLE I.—DIRECTION OF CHANGE IN ANALGESIC ACTION PRODUCED BY MODIFICATIONS OF THE MORPHINE MOLECULE

Modifications of the Morphine Molecule	Direction of Change in Analgesic Action
Phenolic hydroxyl muzzled:	
Codeine or dionin compared to morphine; at position 3 replacement of C—OH by C—OCH_3 or C—OC_2H_5	Decreased to about $^1/_{10}$
Alcoholic hydroxyl muzzled:	
Heterocodeine or 6-monoacetylmorphine compared to morphine; at position 6 replacement of CHOH by $CHOCH_3$ or $CHOCOCH_3$	Increased 2 to 4 times
Saturation of aliphatic ring:	
Dihydromorphine compared to morphine; at position 7–8 replacement of CH=CH by CH_2—CH_2	Usually increased slightly
Alcoholic hydroxyl removed:	
Dihydrodesoxymorphine-D ("Desomorphine") compared to dihydromorphine; at position 6 replacement of CHOH by CH_2	Increased 10 times
Alcoholic hydroxyl replaced by ketone:	
Dihydromorphinone ("Dilaudid") compared to dihydromorphine; at position 6 replacement of CHOH by C=O	Increased 4 to 5 times
Oxygen bridge opened:	
Tetrahydrodesoxymorphine compared to dihydromorphine; position 4 becomes C—OH and position 5 CH_3	Decreased to $^1/_2$ or less
Nitrogen ring opened:	
Methylmorphimethine compared to morphine	Decreased markedly
Tertiary nitrogen changed to quaternary:	
Morphine methochloride compared to morphine; replacement of N—CH_3 by 	Decreased markedly
Nuclear substituent added:	
Aromatic ring—chloromorphine compared to morphine; at position 1 replacement of CH by C—Cl	Decreased markedly
Dihydrohydroxycodeinone compared to dihydrocodeinone;[a] at position 14 replacement of CH by C—OH	Increased
Aliphatic ring—methyldihydromorphine or methyldihydromorphinone ("Metopon") compared to dihydromorphine; at position 7 replacement of CH_2 by 	Variable[b]
Aliphatic ring—6-methyldihydromorphine compared to dihydromorphine; at position 6 replacement of CHOH by CCH_3OH	Usually unchanged, but effect prolonged

[a] Dihydrocodeinone is the codeine analogue of dihydromorphinone.
[b] Effect varies both with the substituent and with the morphine derivative into which it is introduced. Methyldihydromorphine exhibits less, methyldihydromorphinone more analgesic effect than dihydromorphine; ethyldihydromorphinone is about $^1/_2$ and isopropyldihydromorphinone less than $^1/_{10}$ as effective as methyldihydromorphinone.

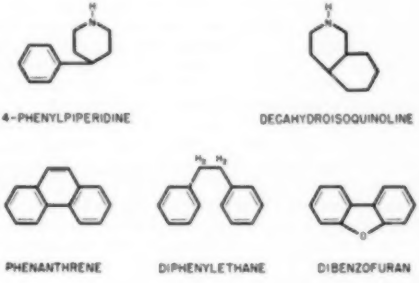


MORPHINE

Figure 1.

deine derived from natural sources. This is a most important accomplishment. It is absolute proof of the morphine-like structure of morphinan and should lead to more complete synthesis of the morphine molecule through closure of the oxygen bridge.

Early in morphine chemistry the phenanthrene nucleus was recognized in the morphine molecule and postulated as a starting material in morphine synthesis. Mosettig and his associates (1) elected to work along this line and found that the addition of an amino alcohol side chain to phenanthrene at a point corresponding to position 3 of the morphine



4-PHENYLPIPERIDINE

DECAHYDROISOQUINOLINE

PHENANTHRENE

DIPHENYLETHANE

DIBENZOFURAN

Figure 2.

molecule resulted in a compound whose analgesic effect was almost as great as that of codeine. The amino alcohol side chain seemed to be the important element. Because its addition to dibenzofuran, an-

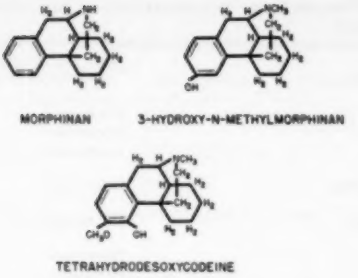


Figure 3.

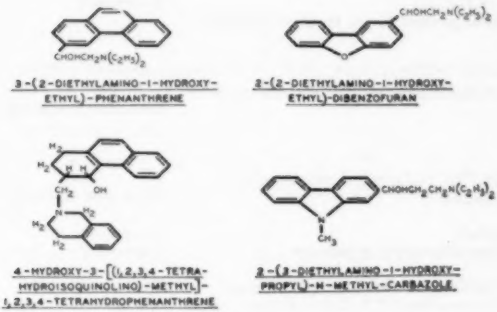


Figure 4.

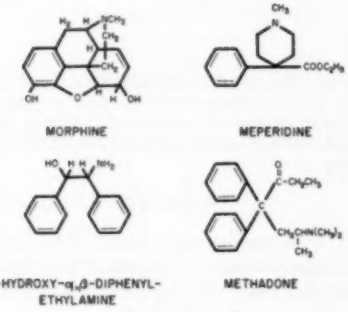


Figure 5.

other moiety of the morphine molecule, or to carbazole, the imino analogue of dibenzofuran, resulted in similar effectiveness (Fig. 4). Still greater analgesic action, as well as some other characteristics of morphine, appeared in a cyclic amino alcohol (Fig. 4) in which partially hydrogenated phenanthrene and isoquinoline nuclei were united (1).

MEPERIDINE AND ITS DERIVATIVES

The nitrogen ring in morphine, if it is closed at carbon 13, corresponds to the structure of piperidine and with the aromatic ring of the molecule may be pictured as a phenylpiperidine (Fig. 2), possibly another basic structure in morphine synthesis. Meperidine or Demerol (Fig. 5) is a substituted phenylpiperidine but speculation on its structural relationship to morphine (6) was *post hoc* reasoning because the demonstration of the analgesic effect of meperidine was initially a by-product of the research which resulted in its synthesis.

As with morphine the meperidine molecule has been subjected to many modifications, both for the accumulation of data on the relation of structure to action and for the production of a more active compound. Many of the changes together with their directional effect on analgesic action are summarized in Table II. Many of these changes were effected by the original investigators (6, 7); most of them have been made by American and English chemists (8–12, 27). "Ketobemidone," the most active compound of the whole series, is thirty times more effective than meperidine as an analgesic, but unfortunately its repeated administration leads to very rapid development of extremely severe physical dependence (addiction) so that its clinical use has been judged inadvisable.

DIPHENYLETHANE DERIVATIVES

Looking once more at the morphine molecule it is possible to see in it a diphenylethane structure (Fig. 2). Dodds and his collaborators (13, 14) investigated a series of diphenylethane derivatives and described a mild analgesic effect with β-hydroxy-α,β-diphenylethylamine (Fig. 5). It is not as active as meperidine and further work along this line has not been promising (15).

METHADONE AND ITS DERIVATIVES

The most striking development in the field of synthetic analgesics came about not by consideration of the morphine molecule but by analogy with work in other fields, and it is difficult to see any direct relation between the structure of the resulting compound, methadone (Fig. 5), and that of morphine. The chemists who first made methadone (7, 16) and many others (17–31) have explored widely the possibilities of modification of the methadone molecule to effect further improvement (Table III). None of the changes, however, has developed greater analgesic activity than in methadone itself, nor, probably, a better compound from the practical standpoint.

OTHER SYNTHETIC ANALGESICS

If one compares the four molecules, morphine, meperidine, methadone, and the diphenylethanolamine (Fig. 5), one finds that the only feature common to all is the presence of a tertiary nitrogen but that the first three also contain a quaternary carbon with a tertiary nitrogen in *beta* relationship to it. At least two attempts have been made recently to use the concept of quaternary carbon and tertiary nitrogen in *beta* relationship as an optimal and perhaps essential feature of potent analgesic agents. Rutenberg and Horning (32) have synthesized oxindoles and Schwartzman (33) has begun work on spirocyclohexyl compounds (Fig. 6). The spirocyclohexyl

TABLE II.—DIRECTION OF CHANGE IN ANALGESIC ACTION PRODUCED BY MODIFICATIONS OF THE MEPERIDINE MOLECULE

Modifications of the Meperidine Molecule	Direction of Change in Analgesic Action
Removal of the phenyl group:	
Replacement of $-C_6H_5$ by $-H$	Decreased
Replacement of $-C_6H_5$ by $-C_2H_5$	Decreased
Replacement of $-C_6H_5$ by naphthyl	Decreased
Shift in position of phenyl group:	
4-Phenylpiperidine, etc., changed to 3-phenylpiperidine, etc.	Decreased
Interpolation of one or more CH_2 groups:	
Replacement of $-C_6H_5$ by $-CH_2C_6H_5$ or $-CH_2CH_2C_6H_5$	Decreased
Addition of substituent to phenyl group:	
Replacement of $-C_6H_5$ by *p*-hydroxyphenyl	Decreased
Replacement of $-C_6H_5$ by *m*-hydroxyphenyl	Increased
Removal of acyloxy group:	
Replacement of $-COOC_2H_5$ by $-H$	Decreased
Replacement of $-COOC_2H_5$ by $-C_6H_5$ or $-CH_2C_6H_5$	Decreased
Substitution of the carboxylate by hydroxyl (change from C—C to C—O linkage) and acylation:	
Replacement of $-\overset{O}{\overset{\|}{C}}-O-C_2H_5$ by $-O-\overset{O}{\overset{\|}{C}}-CH_2CH_3$ (4-(*m*-hydroxyphenyl)-4-propionoxy-1-methylpiperidine)	Increased markedly
Substitution of the carboxylate by ketone:	
Replacement of $-\overset{O}{\overset{\|}{C}}-O-C_2H_5$ by $-\overset{O}{\overset{\|}{C}}-CH_2CH_3$ (4-(*m*-hydroxyphenyl)-1-methyl-4-piperidyl ethyl ketone, "keto-bemidone").	Increased markedly
Shift in position of acyloxy group:	
4-ethylcarboxylate changed to 3-ethylcarboxylate	Decreased
Formation of closed ring structure of phenyl-piperidine-acyloxy linkage	Decreased
Change in alkyl radical on nitrogen:	
Replacement of NCH_3 by NCH_2CH_3 or $NCH_2CH_2CH_3$, etc. (methyl optimal)	Decreased
Addition of substituent to piperidine (4-phenyl-4-propionoxy-1,3-dimethylpiperidine)	Variable[a]
Structural isomerism ("Isopethidine")	Decreased

[a] In the example cited, with methyl added in the 3 position, analgesic effect is increased; other substituents in other positions have decreased activity.

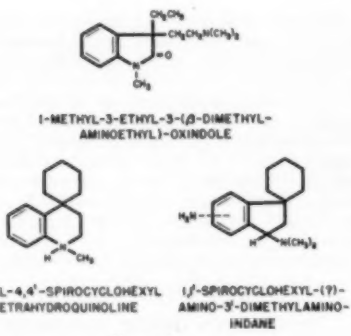


Figure 6.

quinoline compounds are not very stable but do exhibit definite mild analgesic action. The spirocyclohexyl indane shown, on the other hand, is stable and nearly twice as effective as codeine. These results are especially interesting as the outcome of planned synthesis based on consideration of the chemical configuration of previously known analgesics. It is too early to estimate their practical importance.

Analgesic action of some degree has been reported for many other chemical types, summarized briefly in Table IV and illustrated in Figs. 7 and 8. In most instances various other modifications upon the basic structures listed in the table have been made in addition to the ones illustrated in the figures. Effectiveness, however, has not been great, as a rule, and results of practical significance have not yet been forthcoming.

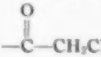
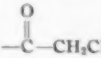
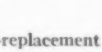
SUMMARY

1. A tertiary nitrogen seems to be essential for analgesic action. It is present in every compound with which a significant degree of analgesic action has been demonstrated.

2. The morphine, meperidine, and methadone molecules each contain a quaternary carbon as well as a tertiary nitrogen and these are connected by a $-CH_2-CH_2-$ linkage. This feature may not be essential for analgesic action but since it is present in the most active compounds it would seem to be desirable and perhaps optimal. Active spirocyclohexyl derivatives have been synthesized as a direct result of the postulation of the desirable nature of the quaternary carbon with an amino nitrogen in *beta* relationship to it.

3. Where a like chemical change has been made in more than one series of compounds it has in each instance had the same qualitative effect on analgesic action so that one can say:

TABLE III.—DIRECTION OF CHANGE IN ANALGESIC ACTION PRODUCED BY MODIFICATIONS OF THE METHADONE MOLECULE

Modifications of the Methadone Molecule	Direction of Change in Analgesic Action
Removal of ketone side chain:	
Replacement of by —H	Decreased markedly
Replacement of  by —OH	Decreased
Replacement of  by —$OCOCH_3$	Decreased[a]
Substitution of ketone:	
Reduction-replacement of by —CH=$CHCH_3$	Decreased
By chlorine-replacement of by	Decreased
By nitrogen-replacement of by	Decreased
By sulfone-replacement of by —SO_2—	Little change
By hydroxyl-replacement of by	Decreased
By hydroxyl and acylation-replacement of by or	[b]
By hydroxyl and aroylation-replacement of by	Decreased markedly
By carboxylate-replacement of  by or	
CH_2CH_3, etc., or by or	Decreased
By carboxylate and formation of amide-replacement of by	Decreased[a]
Lengthening or shortening ketone side chain:	
Replacement of by or	Decreased
Changing symmetrical relationship of phenyl groups; 4,4-diphenylheptanone changed to 4,5-diphenyl heptanone	Decreased
Removal or substitution of one phenyl group:	
Replacement of by or or or	Decreased

(*Continued on p. 250*)

TABLE III.—DIRECTION OF CHANGE IN ANALGESIC ACTION PRODUCED BY MODIFICATIONS OF THE METHADONE MOLECULE (*Continued*)

Modifications of the Methadone Molecule	Direction of Change in Analgesic Action
Substituent on one or both phenyl groups: Replacement of C_6H_5 groups by C_6H_5, *m*-HO—C_6H_4, *m*-CH_3O—C_6H_4, *p*-ClC_6H_4, *p*-ClC_6H_4	Decreased
Structural isomerism, dependent on position of amine: Changing $-CH_2-CH(CH_3)-N(CH_3)_2$ to $-CH(CH_3)-CH_2-N(CH_3)_2$ or $-CH_2-CH_2-CH_2-N(CH_3)_2$ or $-CH(N(CH_3)_2)-CH_2-CH_3$ or $-C(CH_3)_2-N(CH_3)_2$	Decreased
Lengthening or shortening basic side chain: Changing $-CH_2-CH(CH_3)-N(CH_3)_2$ to $-CH_2-CH_2-N(CH_3)_2$ or $-CH_2-N(CH_3)_2$ or $-CH_2-CH(C_2H_5)-N(CH_3)_2$	Decreased
Changing basic group: Replacement of $-N(CH_3)_2$ by $-N$⟨⟩, $-N$⟨⟩O, $-N$⟨⟩, or $-N(CH_3)(CH_2C_6H_5)$	[a]
Changing tertiary to quaternary nitrogen: Replacement of $-N(CH_3)_2$ by $-N(CH_3)_2Cl$	Decreased markedly
Optical isomerism	[e]

[a] Effect much less than with methadone but greater than preceding compound with free hydroxyl.
[b] The acetoxy compound is as effective as methadone, the propionoxy is less effective.
[c] Amide similar to ester, less effective than amidone.
[d] Morpholino and piperidino compounds similar to methadone; others less effective.
[e] The levo isomer is approximately twice as effective as the racemate; the dextro isomer is very much less effective.

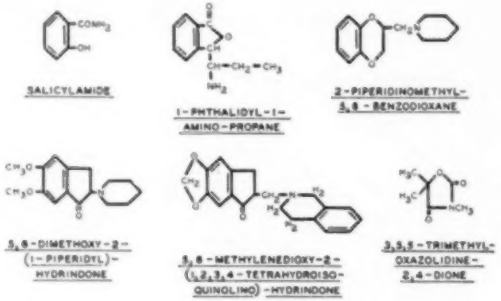


Figure 7.

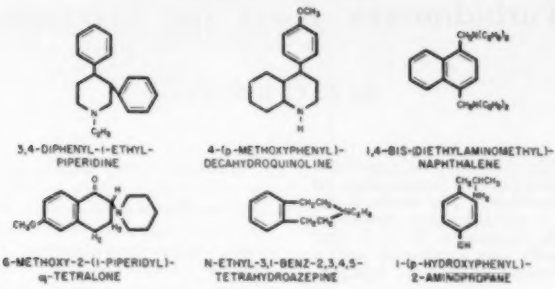


Figure 8.

TABLE IV.—TYPE OF COMPOUNDS EXERTING ANALGESIC ACTIVITY

Type of Compound	Analgesic Activity
Salicylamide (34)	7.5 times acetylsalicylic acid
Phthalidyl derivatives (35)	Less than meperidine
Benzodioxanes (36)	[a]
Hydrindones (37)	[a]
Oxazolidine-2,4-diones (38)	Slight
Diphenyl-ethylpiperidines (39)	Less than meperidine
Phenyl-quinolines (39)	Less than meperidine
Naphthalenes (39)	
6-Methoxy-2-(1-piperidyl)-α-tetralone (40)	[a]
Tetrahydroazepines (41)	Similar to meperidine
2-Amino-6-methylheptane (42)	Greater than codeine
2-Chloropropylamine (31)	Slight
Phenylaminopropanes (43, 44)	

[a] Data on degree of effect not available.
[b] The example illustrated in Fig. 8 is said to be as effective as meperidine.
[c] The analgesic effect of 1-phenyl-2-aminopropane (amphetamine) is slight but that of 1-(*p*-hydroxyphenyl)-2-aminopropane is reported to be greater than that of codeine. (Cf. effect of similar modification in the meperidine series.)

(*a*) that a free acid or its ester is less active than the amide (phenanthrene derivatives particularly) or than a hydroxyl or ketone (morphine, meperidine, and methadone derivatives);

(*b*) that a ketone is a more effective grouping than an alcoholic hydroxyl;

(*c*) that acylation of an alcoholic hydroxyl increases analgesic action;

(*d*) that a tertiary amine is more effective than a secondary or primary;

(*e*) that a change from tertiary to quaternary nitrogen practically abolishes analgesic effect.

4. The various modifications which have been described do not necessarily affect toxicity and analgesic action similarly or in the same direction and, unfortunately, no potent analgesic agent has yet been made which is free of addiction liability.

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A Turbidimetric Assay for Terramycin*

By R. C. KERSEY

A rapid and reliable assay method applicable to both impure and purified preparations of terramycin is described. The method described involves a turbidimetric procedure utilizing *Klebsiella pneumoniae* P. C. I. 602 as the test organism. An incubation period of only three and one-half hours is required.

THE following assay method affords an accurate, convenient and rapid method for determining the potency of terramycin. This antibiotic is produced by an actinomycete isolated from soil and given the name *Streptomyces rimosus* by Finlay, *et al.*, (1). Terramycin has a broad bacterial spectrum which includes both Gram-positive and Gram-negative organisms and has a low toxicity *in vivo*. The procedure embodies the test organism and media for the streptomycin turbidimetric assay as recommended by the Food and Drug Administration (2).

PROCEDURE

Culture and Inoculum.—Stock cultures of *Klebsiella pneumoniae* P. C. I. 602 are carried on agar slants of the following composition: Beef extract 1.5 Gm., yeast extract 3.0 Gm., peptone 6 Gm., agar 15 Gm., distilled water 1 liter. The *p*H of the medium is adjusted to 7.0.

The assay inoculum is prepared by transferring the organism to fresh agar slants and incubating for six hours at 37°. The growth from two or three slants is suspended in sterile distilled water and 5.0 ml. of the suspension added to each of two Roux bottles containing the agar described above. The Roux bottles are incubated at 37° for six hours, the growth harvested and sufficient sterile distilled water added to give a light transmission reading of 80% using a 650 mμ filter in a photoelectric colorimeter. This suspension may be stored for two weeks.

Assay Medium.—The medium used in the assay is composed of: Peptone 5.0 Gm., yeast extract 1.5 Gm., beef extract 1.5 Gm., sodium chloride 3.5 Gm., glucose 1.0 Gm., KH_2PO_4 1.32 Gm., K_2HPO_4 3.68 Gm., distilled water 1 liter. The medium is adjusted to *p*H 7.0 and sterilized. This medium may be obtained in dehydrated form.[1]

Standard Solution.—A stock solution is prepared by dissolving 25.0 mg. of crystalline terramycin base in 100 ml. of 0.1 *N* HCl. This stock solution may be stored in the refrigerator for a period of five days with no loss in potency. Final dilutions of the standard are prepared daily by diluting the stock solution to 2.5 μg./ml. with distilled water.

Samples.—Liquid samples are diluted with water to contain from 0.5 to 2.5 μg./ml. of terramycin. Dry samples of the free base, which is not readily soluble in water, are more easily handled by first dissolving in a small amount of 0.1 *N* HCl and then making the required dilution with water. Urine samples are centrifuged if turbid and the proper dilutions made with water. Samples of whole blood are centrifuged or allowed to clot and dilutions made on the clear serum.

Assay Techniques.—The general assay procedure is based on McMahan's turbidimetric assay of penicillin (3). The preparation of the standard curve and sample series is described below.

A standard curve (0.05 to 0.5 μg./tube) is prepared by pipetting, with a micro-pipette (3), the following amounts of the standard terramycin solution into a series of 15 x 100-mm. Pyrex test tubes: 0.20, 0.18, 0.16, 0.14, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02 ml. In a further series the diluted samples (0.5 to 2.5 μg./ml.) are pipetted as follows: 0.20, 0.16, 0.12, 0.08, 0.04 ml. The small volume of sample employed (0.02 to 0.20 ml.) makes an insignificant difference in the final volume of medium in the tube (7 ml.) and hence no precaution need be taken to adjust all the tubes to the same volume.

A flask containing sufficient sterile assay broth to fill all tubes in the test series is inoculated with 6 ml. of the standardized *Klebsiella pneumoniae* cell suspension per 100 ml. of broth. A Brewer automatic pipetting machine is used to dispense 7 ml. of the inoculated medium to each tube, the tubes covered with inverted stainless steel trays and incubated at 37° for three and one-half hours in a water bath.

The racks are removed at the end of the incubation period, and steamed for ten minutes in an Arnold sterilizer to prevent additional growth from taking place during the reading of the test.

Turbidity readings are made with a Lumetron colorimeter, Model 402E, and light transmission is read directly from the galvanometer. The sensitivity of the colorimeter is adjusted so that a galvanometer deflection of 80 divisions is obtained between the high and low points of the standard curve. Galvanometer readings are plotted against potency and the points connected in a smooth curve as illustrated in Fig. 1. Calculations of a typical assay are given in Table I.

RESULTS

A reliable assay is dependent upon: (*a*) consistency of values when potencies are calculated from various portions of the standard curve (see Table I, Column *c*/*a*); (*b*) recovery experiments; and (*c*), reproducibility of assays on successive days. Data are presented in Table I, Column *c*/*a* demonstrating that the small variations noted at different portions of the standard curve are within the limits of other turbidimetric assays. Recovery experiments with human and dog urine demonstrate that urines do not interfere with the method (see Table II).

* Received March 23, 1950, from the Biochemical Research Laboratories, Chas. Pfizer and Co., Inc., Brooklyn, N. Y.

[1] Baltimore Biological Laboratories, Inc., Baltimore, Md., Antibiotic Assay Broth.

TABLE I.—TERRAMYCIN ASSAY AND CALCULATIONS

Volume of Standard, Ml.	Terramycin/ Tube, μg.	Galvanometer Reading
0.20	0.50	90
0.18	0.45	85
0.16	0.40	80
0.14	0.35	79
0.12	0.30	70
0.10	0.25	60
0.08	0.20	50
0.06	0.15	39
0.04	0.10	28
0.02	0.05	20
0.00	0.00	13

Volume of Sample, Ml. (a)	Galvanometer Reading (b)	Terramycin Calculated from Curve, μg. (c)	Terramycin, μg. per Ml. Diluted Sample (c/a)	Average (d)[a]
0.20	84	0.430	2.15	2.08
0.16	73	0.325	2.03	
0.12	60	0.250	2.09	
0.08	42	0.165	2.06	

[a] $d \times$ dilution = potency of sample.

TABLE II.—RECOVERY OF TERRAMYCIN FROM BLOOD AND URINE[a]

	Calculated, μg./Ml.	Found, μg./Ml.	Recovery, %
	Urine		
Human	50	52	104
Control	0	0	...
Dog	50	54	108
Control	0	0	...
	Blood		
Human	25	25.5	102
Control	0	0	...
Dog	25	27	108
Control	0	0	...

[a] All values are an average of three assays.

Reproducibility of the assay on different days is demonstrated by the data in Table III. Representative samples of terramycin base and hydrochloride were analyzed on each of three successive days. Assays were reproducible within 2%.

The method has been applied successfully on widely different types of samples including fermentation broths, samples from the recovery process, blood, and urine. The applicability of the method to other biological fluids is being investigated.

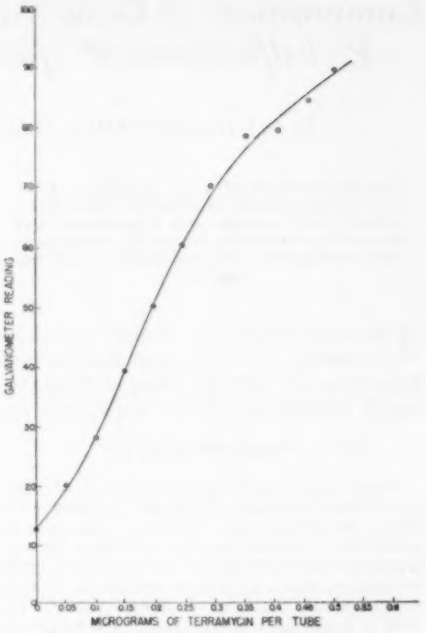

Fig. 1.—Terramycin dose response curve.

TABLE III.—REPRODUCIBILITY OF ASSAYS ON DIFFERENT DAYS[a]

	μg./Ml.		
	1st Day	2nd Day	3rd Day
Terramycin base	915	930	915
Terramycin HCl	861	861	847

[a] Average of three assays.

SUMMARY

A method is described for the turbidimetric assay of terramycin employing *Klebsiella pneumoniae* P. C. I. 602 as the assay organism. An incubation period of three and one-half hours is required. The method is rapid, reliable, and applicable to both impure and purified preparations.

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Composition of Gum Turpentines of *Pinus aristata, P. balfouriana, P. flexilis,* and *P. parviflora**,†

By A. J. HAAGEN-SMIT, TIAO-HSIN WANG, and N. T. MIROV

The composition of gum turpentines from each of four species of *Pinus* has been determined. Each sample was fractionated and the per cent of volatile oil and its components was investigated. The results of the study are reported.

THE PRESENT paper is a continuation of an investigation (1) on the composition of gum turpentines and deals with those of *Pinus aristata, P. balfouriana, P. flexilis,* and *P. parviflora.*

PINUS ARISTATA

Pinus aristata grows in high altitudes of desert ranges from Colorado to California, but only in the White Mountains and Panamint Mountains in California. It is not found in the Sierra Nevada. Turpentine for this study was collected in the White Mountains, which lie east of the Sierra Nevada, separated from the latter by Owens Valley. The yield of turpentine was very small. The turpentine has the following constants: d_4^{25}, 0.8649; n_D^{25}, 1.4656; $[\alpha]_D^{25}$, +25.21.

A sample of 150 Gm. of the turpentine was fractionated (Table I); more than 80% of the oil distilled over at a temperature between 154–160°. Physical constants indicated the presence of α-pinene, which according to Wallach (2) has the following constants: b. p. 155–156° at 1 atm.; d^{20}, 0.858; n_D^{20}, 1.4655. This was confirmed by the preparation of α-pinene nitrosochloride from fraction 2 according to the procedure of Wallach (2). The nitrosochloride thus obtained melted at 108–109° after being purified by washing with ethyl alcohol and by dissolving in chloroform and subsequent precipitation with methyl alcohol. It gave no depression in melting point when added to an authentic sample of the compound which, according to Simonsen (3), has a melting point varying considerably according to the method of purification and with the rate of heating but, as a general rule, ranging from 105° to 108°.

The boiling point ranges of fractions 7 and 9 are close to those of β-pinene and Δ³-carene, respectively, but the values of density and refractive index remained close to those of α-pinene. Attempts were made to prepare, from fractions 7 and 9, nopinic acid by oxidation with alkaline potassium permanganate, and Δ³-carene nitrosate by treatment in acetic acid solution with amyl nitrite and nitric acid; these attempts failed. However, both fractions 7 and 9 gave good yields of α-pinene nitrosochloride, with m. p. of 108–108.5°, when Wallach's procedure was applied.

Distillation behavior indicated the accumulation of a pure component in fraction 14 with physical characteristics of a sesquiterpene. A determination of the molecular refraction (M_D, 62.81) revealed the sesquiterpene to be tricyclic and hence containing one double bond. A solution of the oil in absolute ether was saturated with dry HCl gas at 0° and allowed to stand overnight. Upon evaporation of the solvent, the oily residue was allowed to stand at room temperature for about five weeks, when crystalline hydrochloride separated out as plates. After recrystallization five times from ethyl alcohol and water, the hydrochloride melted at 80–80.5°. No sesquiterpene described in the literature could be found to form a hydrochloride of this melting point.

By estimation from the per cent of distillate at various temperature ranges, it may be concluded that the turpentine of *P. aristata* contains mainly (96%) of *dl-* and *d*-α-pinene, with about 4% of a tricyclic sesquiterpene of unknown structure but which forms a hydrochloride melting at 80.5°.

PINUS BALFOURIANA

From Northern Location

Pinus balfouriana (foxtail pine) is a rare alpine, 5-needle, white pine, growing in two widely separated areas in California. In the northern part of the state, foxtail pine grows in patches on several mountain ridges at an elevation of 7000 to 8000 feet (Scott Mountains, Marble Mountains, Salmon Mountains, Trinity Alps, and Yolla Bolly Mountains). The southern location is in the southern high Sierra Nevada at 9000 to 12,000 feet.

The oleoresin for this study was collected during July, 1947, in the Scott Mountains, Siskiyou County, Calif., at an elevation of 7500 feet by staff members of the Institute of Forest Genetics. The oleoresin had a "typical" white pine honeylike consistency, and the resin acids crystallized very reluctantly on standing. Altogether 5170 Gm. of oleoresin was obtained in three weeks from 70 mature trees. Poor yield apparently was due to the cold weather prevailing during the first half of July.

Yield of turpentine from the oleoresin was very high, 28.3%. The turpentine had the following characteristics: d_4^{25}, 0.8570; n_D^{25}, 1.4643; $[\alpha]_D^{25}$, −11.05°.

A sample of foxtail turpentine was fractionated and the results are shown in Table II. The main part of the oil distilled over at 154–160°, having physical constants close to those of α-pinene. Its identification was confirmed by the preparation of α-pinene nitrosochloride, having a melting point of 108° and giving no depression in melting point with an authentic sample of the compound.

The physical constants of fractions 6 and 7 indicate the presence of *l*-β-pinene in addition to α-

* Received October 24, 1949, from the U. S. Dept. of Agriculture Forest Service, Berkeley, Calif.

† Joint contribution from the Kerckhoff Laboratories of Biology, California Institute of Technology, and the California Forest and Range Experiment Station, which is maintained by the Forest Service, U. S. Dept. of Agriculture, in cooperation with the University of California.

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINE OF *Pinus aristata*

Fraction No.	Distillation Range: Temperature °C.	Distillation Range: Pressure, Mm.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	130–154	747	1.3	...	...	...
2	154–156	747	46.3	0.854	1.4642	+26.12°
3	156–158	747	31.4	...	...	...
4	158–160	747	6.7	...	...	...
5	160–162	747	1.7	...	...	...
6	162–164	747	1.8	...	...	...
7	164–166	747	2.0	0.857	1.4680	+20.89°
8	166–168	747	0.5	...	...	...
9	168–170	747	2.0	0.854	1.4684	+19.31°
10	60–74	17	0.5	...	...	...
11	74–100	17	1.0	...	...	...
12	100–110	17	0.4	...	...	...
13	110–118	17	0.3	...	...	...
14	118–120	17	1.3	0.929	1.4840	...
15	Residue and loss	...	2.8	...	...	...

TABLE II.—FRACTIONAL DISTILLATION OF TURPENTINE OF *Pinus balfouriana* (FROM NORTHERN LOCATION)

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	116–154	740	0.7	...	...	...
2	154–156	740	76.6	0.8508	1.4634	−10.23°
3	156–158	740	10.0	...	...	...
4	158–160	740	3.8	...	...	...
5	160–162	740	0.4	...	...	...
6	162–164	740	0.7	...	...	...
7	164–166	740	1.1	0.8485	1.4720	−27.03°
8	166–172	740	0.4	...	...	...
9	172–174	740	0.4	...	...	...
10	174–176	740	0.6	0.8441	1.4720	−53.25°
11	176–178	740	0.4	...	...	...
12	178–184	740	0.5	...	...	...
13	184–190	740	0.3	...	...	...
14	100–135	75	0.3	...	...	...
15	135–145	75	0.7	...	...	...
16	145–154	75	1.0	0.9241	1.4940	+12.52°
17	154–156	75	0.8	0.9171	1.4962	+13.85°
18	156–159	75	0.6	0.9161	1.4985	+13.81°
19	Residue and loss	...	0.7	...	...	...

pinene. Nopinic acid was prepared from fraction 7 by oxidation with alkaline potassium permanganate according to the method of Wallach (4); it melted at 126° and gave no depression in melting point with an authentic sample of nopinic acid, which is reported (5) to melt at 126–127°.

Fractions 10 and 11 have properties very close to those of *l*-limonene as shown in Table III. A sample of fraction 11 was dissolved in 1:1 ethanol-diethyl ether and saturated with bromine at 0° according to Wallach (6). The tetrabromide melted at 118–119° and gave no depression in melting point with an authentic sample of *dl*-limonene tetrabromide.

TABLE III.—CONSTANTS OF *l*-LIMONENE AND FRACTION 10

Property	*l*-Limonene[a]	Fraction 10
Boiling point, °C.	176–176.4° at 1 atm.	174–176° at 740 mm.
Density	d_4^{25}, 0.8422	d_4^{25}, 0.8441
Index of refraction	n_D^{25}, 1.4747	n_D^{25}, 1.4720
Specific rotation	−122.6°	−53.18°
Per cent carbon	88.24 calcd. for $C_{10}H_{16}$	87.38 found
Per cent hydrogen	11.76	12.09

[a] Baeyer, A., *Ber.*, **27**, 448(1894).

The high boiling of oil fractions 16, 17, and 18 (Table IV) possessed the characteristic constants of a sesquiterpene; it did not form a solid hydrochloride. The percentage composition of carbon and hydrogen agreed well with those of $C_{15}H_{24}$ and molecular refraction indicated that it was a tricyclic sesquiterpene with one double bond. The only sesquiterpene recorded in the literature having physical constants close to those described for fractions 16, 17, and 18 was aromadendrene (7), which upon ozonization in acetic acid solution yielded a crystalline ketone, aromadendrone, melting at 80–81°. A sample of oil from fraction 18 was ozonized according to the procedure described by Briggs and Short (7), but no crystalline product was obtained. Thus although the oil of fractions 16–18 is a sesquiterpene containing three rings and one double bond and its physical constants agree very closely with those of aromadendrene, its identification is uncertain.

In conclusion, it may be stated that the turpentine oil of *P. balfouriana* consists mainly of *dl*- and *l*-α-pinene (90%) with some *dl*- and *l*-β-pinene (2%), *dl*- and *l*-*limonene* (2%) and a tricyclic sesquiterpene (4%) of unknown structure.

TABLE IV.—CONSTANTS OF FRACTIONS 16–18 AND AROMADENDRENE

Property	Fraction 16	Fraction 17	Fraction 18	Aromadendrene[a]
Boiling point, °C.	145–154° at 75 mm.	154–156° at 75 mm.	156–159° at 75 mm.	121° at 10 mm.
Density	d_4^{25}, 0.9214	d_4^{25}, 0.9171	d_4^{25}, 0.9161	d_4^{25}, 0.9116
Index of refraction	n_D^{25}, 1.4940	n_D^{25}, 1.4962	n_D^{25}, 1.4985	n_D^{25}, 1.4978
Molecular refraction	64.45	65.00	65.30	65.58
Per cent carbon	85.32 found	...	87.45 found	88.23 calcd. for
Per cent hydrogen	11.90 found	...	12.04 found	11.77 calcd. for $C_{15}H_{24}$

[a] Briggs and Short (7).

From Southern Location

The oleoresin of *P. balfouriana* growing in the southern location was collected at Onion Valley in the Sierra Nevada, just opposite the town of Independence at an elevation of 9000 feet, in the summer of 1948. It is interesting that while the turpentine from the northern location is levorotatory, that from the southern location is dextrorotatory and has the following characteristics: d_4^{25}, 0.8612; n_D^{25}, 1.4642; $[\alpha]_D^{25}$, + 24.33.

A sample of 250 Gm. of the turpentine was fractionated (Table V) and its major part distilled over at 153–157°, possessing the physical characteristics of α-pinene. Identification was confirmed by preparation of α-pinene nitrosochloride from fraction 2 and it melted at 108–109° (2). Fraction 8 has a boiling-point range close to that of β-pinene and Δ^3-carene, but attempts to prepare nopinic acid or Δ^3-carene nitrosate failed. However, fraction 8 gave good yield of α-pinene nitrosochloride, when treated with amyl nitrite and hydrochloric acid (2).

Fraction 14 indicated the accumulation of a pure component; it had a pleasant odor strongly suggesting that of an ester. Saponification test was applied with a positive result. Upon distillation of the saponified liquid, droplets of oil could be observed in the distillate, from which a derivative, 3,5-dinitrobenzoate, melting at 154°, was prepared. It gave no depression in melting point when added to borneol 3,5-dinitrobenzoate, which has a melting point of 154° as recorded in literature (8). After the volatile alcohol was distilled off, the saponified liquid was acidified with sulfuric acid and again distilled The distillate was acidic to litmus; it was neutralized with NaOH solution and evaporated to dryness. From the residue *p*-phenyl phenacyl ester was prepared and after being purified by passing through a chromatographic column (9), the flaky colorless crystals melted at 109–110° and gave no depression in melting point when mixed with an authentic sample of *p*-phenyl phenacyl acetate, melting at 111° (10). Thus the acid was acetic acid and the ester in fraction 14 was bornylacetate.

Fraction 16 has physical constants close to those of a sesquiterpene; its molecular refraction, 63.1, indicated that the sesquiterpene was tricyclic with one double bond. It formed no solid hydrochloride.

The composition of turpentine of *P. balfouriana* from the southern location is thus composed mostly of *dl*- and *d*-α-pinene (98%) with 1% each of borneol-acetate and a tricyclic sesquiterpene. One point of interest is that the two samples of turpentine of the same species, *P. balfouriana*, but from two different and isolated localities, differ from each other not only in the sign of optical rotation, but in chemical composition as well.

PINUS FLEXILIS

Pinus flexilis, commonly known as limber pine, grows on the upper slopes of the Rocky Mountains of Alberta and Montana and at high elevations of mountain ranges in Nevada, Arizona, New Mexico, and southern California. The oleoresin sample was obtained from scattered trees growing at an elevation of 10,000 feet in the White Mountains, Inyo County, Calif.

In distilling the oleoresin of limber pine, great difficulty was experienced in expelling the higher

TABLE V.—FRACTIONAL DISTILLATION OF TURPENTINE OF *Pinus balfouriana* (FROM SOUTHERN LOCATION)

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	151–153	750	5.9	...	...	...
2	153–155	750	43.8	0.8559	1.4635	+23.72°
3	155–157	750	32.6	...	...	...
4	157–159	750	5.7	...	...	...
5	159–161	750	4.0	...	...	...
6	161–163	750	2.0	...	...	...
7	60–62	17	0.8	...	...	...
8	62–64	17	0.8	0.8634	1.4650	+19.11°
9	64–71	17	0.9	...	...	...
10	71–80	17	0.3	...	...	...
11	80–90	17	0.3	...	...	...
12	90–98	17	0.2	...	...	...
13	98–106	17	0.2	...	...	...
14	106–110	17	0.4	0.9010	1.4732	+14.42°
15	110–125	17	0.5	...	...	...
16	125–130	17	0.3	0.9318	1.4880	+32.36°
17	Residue and loss	...	1.3	...	...	...

boiling fractions of turpentine. At 0.05 mm. of pressure with the flask temperature reaching 210°, the residue (rosin) still remained soft. Twenty per cent of turpentine was obtained. The turpentine possessed the following characteristics: d_4^{25}, 0.8606; n_D^{25}, 1.4707; $[\alpha]_D^{25}$, +41.2°.

A sample of 350 Gm. of the turpentine was fractionated; the majority of the oil distilled over at 154–160° at 750 mm. and at 90–100° at 17 mm. (Table VI). The physical constants of the oil distilling at these temperature ranges are close to those of α-pinene, and its identification was confirmed by the preparation of a derivative, α-pinene nitrosochloride, from fractions 3 and 10 which, after purification, melted at 108–109° and gave no depression in melting point when added to an authentic sample of the compound.

The absence of β-pinene and Δ^3-carene in these fractions was demonstrated by the failure to yield nopinic acid upon oxidation with alkaline $KMnO_4$ or to yield Δ^3-carene nitrosate when treated with amyl nitrite and nitric acid.

Fraction 15 has the physical constants of a sesquiterpene and has a value of 65.6 for molecular refraction, indicating the sesquiterpene to be bicyclic with two double bonds. Upon dehydrogenation of fraction 15 with sulfur (11) cadalene was formed which was characterized by the formation of a picrate melting at 115° (11). No azulene could be isolated from the dehydrogenation products. The sesquiterpene formed no solid hydrochloride and did not react with maleic anhydride, showing the absence of conjugation of the double bonds.

Fraction 24 possessed a molecular refraction of 67.9 and other physical constants close to those of monocyclic sesquiterpene with three double bonds. It formed no solid hydrochloride, but upon dehydrogenation with sulfur (11) it gave cadalene, which after distillation over metallic sodium formed a picrate melting at 115–116°. No azulene could be isolated from the dehydrogenation products. Fraction 24 also formed an addition compound with maleic anhydride (12) and after recrystallization from alcohol, colorless needle-shaped crystals were obtained. The crystals melted at 149–150° and gave no depression in melting point with albicaulene maleic anhydride addition product (13). These reactions indicated that the sesquiterpene is albicaulene.

In conclusion, it may be said that the turpentine of *P. flexilis* is mainly composed of *dl*- and *d*-α-pinene (80%) with about 13% of albicaulene, a monocyclic sesquiterpene, and 7% of a bicyclic sesquiterpene of unknown structure.

PINUS PARVIFLORA

Pinus parviflora is a five-needle (white) pine, i.e., of the section Haploxylon. It grows in the mountains of Japan and Formosa. A large sample of oleoresin was obtained through the courtesy of the Japanese Bureau of Forestry. The oleoresin was collected during a period from August 7 to October 1, 1947, from 50 trees, 30 cm. in diameter, growing at an elevation of about 3000 feet, near Andon Village, Andon National Forest, Nagano Prefecture, Japan.

When a sample of oleoresin was distilled under 0.2 mm. pressure at flask temperature reaching 175°, the yield of turpentine was 20% and the kettle residue (rosin) was rather soft. With an increase of tem-

TABLE VI.—FRACTIONAL DISTILLATION OF TURPENTINE OF *Pinus flexilis*

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	90–152	750	1.9	...	...	...
2	152–154	750	3.7	...	...	...
3	154–156	750	30.0	0.8499	1.4634	+30.4°
4	156–158	750	22.5	...	...	...
5	158–160	750	8.5	...	...	...
6	53–60	17	0.3	...	...	...
7	60–80	17	1.0	...	...	...
8	80–90	17	1.2	...	...	...
9	90–97	17	2.3	...	...	...
10	97–100	17	8.9	0.8439	1.4620	+25.7°
11	100–110	17	0.3	...	...	...
12	110–120	17	0.3	...	...	...
13	120–130	17	0.6	...	...	...
14	130–138	17	0.3	...	...	...
15	138–140	17	1.9	0.8942	1.4872	+34.9°
16	140–144	17	0.3	...	...	...
17	144–146	17	0.4	...	...	...
18	146–148	17	0.3	...	...	...
19	148–150	17	0.6	...	...	...
20	90–110	0.05	0.4	...	...	...
21	110–120	0.05	0.4	...	...	...
22	120–136	0.05	0.7	...	...	...
23	136–140	0.05	2.4	...	...	...
24	140–142	0.05	3.9	0.9017	1.5120	...
25	142–150	0.05	1.3	...	...	...
26	150–160	0.05	0.7	...	...	...
27	160–200	0.05	0.3	...	...	...
28	200–205	0.05	1.7	Solidified on cooling		
29	Residue and losses	...	3.0	...	...	...

perature to 210° toward the end of distillation of 0.2 mm. pressure, 28% of turpentine was obtained and the residue was hard and brittle.

The turpentine possessed the following constants: d_4^{25}, 0.8880; n_D^{25}, 1.4858; $[\alpha]_D^{25}$, +17.66.

A sample of 300 Gm. of turpentine from *P. parviflora* was fractionated with results shown in Table VII. The constants of fractions 2 and 3 indicated the presence of *l*-α-pinene. This was confirmed by the preparation of a solid derivative, α-pinene nitrosochloride, which melted at 108° and gave no depression in melting point when mixed with an authentic sample of the same compound. Fractions 6–8 which could contain, in addition to α-pinene, some β-pinene and Δ^3-carene, did not yield nopinic acid or Δ^3-carene nitrosate.

Fraction 11 represented another maximum in the temperature-weight distribution graph and hence accumulation of a component. Microanalysis gave 81.53% of carbon and 11.22% of hydrogen, indicating the presence of 7.25% of oxygen. A saponification test was applied with positive results. The saponified liquid was acidified and the volatile acid distilled off and identified to be acetic acid by the determination of its Duclaux constants (14). The residue, after removal of the volatile acid, was extracted with ether and upon evaporation of the solvent, a yellow oil having an odor recalling that of borneol was obtained. From the oil a 3,5-dinitrobenzoate was prepared. It was obtained as needle-shaped crystals melting at 95° after recrystallization from petroleum ether. From the saponification values, it was calculated that fractions 11 and 12 contained approximately 54% of the ester; fractions 13 and 14, 30% each, and fraction 15, 22%.

The boiling-point range (252–253° at 740 mm.) and other physical constants of fraction 18 suggested the presence of a sesquiterpene. It had a molecular refraction of 65.5; that calculated for $C_{15}H_{24}F_2$ is 66.1, showing that the hydrocarbon was a bicyclic sesquiterpene containing two double bonds. By saturating an absolute ether solution of the oil at 0° with dry hydrogen chloride, a dihydrochloride, melting at 107–108° after recrystallization from alcohol, was obtained. The dihydrochloride depressed the melting point of cadinene hydrochloride, thus eliminating the possibility of the hydrocarbon being cadinene. Microanalysis gave 64.92% of carbon, 9.95% of hydrogen, and 25.15% of chlorine; calculated for $C_{15}H_{24}$: 2HCl, carbon is 64.97%, hydrogen, 9.49%, and chlorine, 25.58%.

Fraction 15 also formed a dihydrochloride, melting at 107°, and it gave no depression in melting point when mixed with the dihydrochloride of fraction 18. This indicated that fraction 15 was a mixture of this sesquiterpene and the acetic acid ester Similarly, fractions 11, 12, 13, and 14 were mixtures of the same components but of varying proportions.

Fraction 21 has constants close to those of cadinene (15). These constants are tabulated in Table VIII. The identification was further confirmed by the preparation of the dihydrochloride derivative, which melted at 117–118° and which gave no depression in melting point with an authentic sample of cadinene dihydrochloride, melting at 117°.

The oil of fraction 22 was slightly greenish blue in color. It did not form a solid hydrochloride when an ether solution of the oil was saturated with dry hydrogen chloride, thus showing the absence of any considerable quantity of cadinene. Upon dehydrogenation with selenium (11), two products were isolated and identified; a blue hydrocarbon, azulene (16), ($C_{15}H_{18}$), which formed a picrate melting at 120°; and cadalene, characterized by the formation of a picrate melting at 113°. The yield of azulene

TABLE VII.—FRACTIONAL DISTILLATION OF TURPENTINE OF *Pinus parviflora*

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Distillate, %	Density, $d_4^{25°}$	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	66–68	30	0.7	...	...	...
2	68–70	30	23.4	...	...	...
3	70–72	30	15.0	0.8528	1.4654	−10.26°
4	72–74	30	7.0	...	...	...
5	74–86	30	1.0	...	...	...
6	86–90	30	0.5	...	...	...
7	90–94	30	0.7	0.8586	1.4702	−14.50°
8	94–100	30	0.7	...	...	...
9	100–110	30	0.5	...	...	...
10	110–124	30	0.7	...	...	...
11	124–128	30	1.2	0.9079	1.4701	−5.73°
12	128–138	30	0.5	...	...	...
13	138–140	30	1.1	...	...	...
14	140–142	30	1.2	...	...	...
15	142–144	30	2.5	...	...	...
16	144–146	30	2.3	...	...	...
17	146–148	30	0.8	...	...	...
18	148–150	30	6.0	0.9102	1.4958	+23.35°
19	150–152	30	5.5	...	...	...
20	152–154	30	1.7	...	...	...
21	134–136	16	6.3	0.9154	1.5038	+38.34°
22	136–138	16	10.5	0.9125	1.5150	+117.6°
23	138–150	16	1.0	...	...	...
24	150–160	16	0.8	...	...	...
25	160–178	16	1.0	...	...	...
26	Residue and losses	...	7.4	...	...	...

TABLE VIII.—CONSTANTS OF FRACTION 21 AND CADINENE

Property	Fraction 21	Cadinene[a]
Boiling point, °C.	265–266° at 740 mm. 134–136° at 16 mm.	134–136° at 11 mm.
Density	d_4^{25}, 0.9154	d_4^{25}, 0.9189
Index of refraction	n_D^{25}, 1.5038	n_D^{25}, 1.5079
Molecular refraction	65.97	66.13
Analysis	% C, 87.29 % H, 12.06 found	% C, 88.23 % H, 11.77 calcd. for $C_{15}H_{24}$
Dihydrochloride		
M. p., °C.	117–118°	117°
Mixed m. p., °C.	117–118°	...

[a] Henderson and Robertson (15).

was approximately 10%, while that of cadalene, 30% of the weight of the oil used in the dehydrogenation test. The oil of fraction 22 also formed an addition product with maleic anhydride, thus indicating the presence of a conjugated system of double bonds in the molecule. The maleic anhydride addition product melted at 150° and did not show a depression in melting point when mixed with an authentic sample of maleic anhydride addition product of albicaulene. Albicaulene (13) is a sesquiterpene recently found in the turpentine of *P. albicaulis*. Albicaulene also formed cadalene when dehydrogenated by heating with sulfur or selenium but did not give an azulene under these circumstances. These facts show that fraction 22 is a mixture of albicaulene and a second sesquiterpene, which upon dehydrogenation gives azulene. The greenish tint of fraction 22 shows that traces of azulene are present in the fraction. The physical constants of albicaulene and those of fraction 22 are given in Table IX.

In conclusion, it may be stated that the composition of turpentine of *P. parviflora* is composed of *dl*- and *l*-α-pinene (50%), an acetic ester of a terpene alcohol (2%), cadinene (8%), a bicyclic sesquiterpene of unknown structure (25%) and albicaulene together with a hydrocarbon which gave azulene upon dehydrogenation (15%). The new bicyclic sesquiterpene gives a dihydrochloride, m. p. 107°, and cadelene upon dehydrogenation.

TABLE IX.—CONSTANTS OF ALBICAULENE AND FRACTION 22

Property	Albicaulene[a]	Fraction 22
Boiling point, °C.	180° at 20 mm.	136–138° at 0.5 mm.
Density, d_4^{25}	0.9195	0.9125
Index of refraction, n_D^{25}	1.5190	1.5150
Molecular refraction	67.96	67.42
Molecular refraction calcd. for $C_{15}H_{24}$	67.86	....
Picrate of dehydrogenation product, m. p.	113°	113°
Maleic anhydride addition product		
M. p., °C.	149°	150°
Mixed m. p., °C.	150°	...

[a] Haagen-Smit, Wang, and Mirov (13).

SUMMARY

In the investigation reported here, the turpentines of four species of pine have been studied and found to have the following compositions:

Pinus aristata, 96 per cent of *dl*- and *d*-α-pinene and 4 per cent of a tricyclic sesquiterpene of unknown structure.

P. balfouriana (northern location), 90 per cent of *dl*- and *d*-α-pinene, 2 per cent of *dl*- and *l*-β-pinene, 2 per cent of *dl*- and *l*-limonene, and 4 per cent of a tricyclic sesquiterpene.

P. balfouriana (southern location), 98 per cent of *dl*- and *d*-α-pinene and 1 per cent each of bornel acetate and a tricyclic sesquiterpene.

P. flexilis, 80 per cent of *dl*- and *d*-α-pinene, 13 per cent of albicaulene, a monocyclic sesquiterpene, and 7 per cent of a bicyclic sesquiterpene of unknown structure.

P. parviflora, 50 per cent of *dl*- and *l*-α- pinene, 2 per cent of an acetic ester of a terpene alcohol, 8 per cent of cadinene, 25 per cent of a bicyclic sesquiterpene of unknown structure, and 15 per cent of a mixture of albicaulene and a hydrocarbon which gives azulene upon dehydrogenation with sulfur.

It is of interest to note that the same species of *P. balfouriana* from two different and isolated localities yield turpentines of different chemical composition. α-Pinene is the only constituent common to both, and yet it differs in two localities in the sign of optical rotation.

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Composition of Gum Turpentines of Pines: A Report on *Pinus ponderosa, P. banksiana, P. canariensis,* and *P. washoensis*[*,†]

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The oleoresins obtained from *Pinus ponderosa, P. banksiana, P. canariensis,* and *P. washoensis* have been investigated. The per cent and composition of volatile oil in each oleoresin have been determined and are reported.

THE PRESENT paper is a continuation of an investigation (1) of the oleoresin present in species of the genus *Pinus* and deals with composition of the following pines: *Pinus ponderosa, P. banksiana, P. canariensis,* and *P. washoensis.*

PINUS PONDEROSA

Pinus ponderosa is a widely distributed species and is variable in its external characteristics. It grows on the Pacific Coast from British Columbia to California. Its Rocky Mountain form, which merges into the Pacific Coast form in the north and grows in the Black Hills of South Dakota, and northwest Nebraska, descends as far south as Arizona and western Texas.

The composition of the gum turpentine of *P. ponderosa* from California has previously been reported by Schorger (2), as consisting of 5% *l*-α-pinene, 60% *l*-β-pinene, 20% limonene, and 10% cadinene. However, since Schorger had carried out his investigation previous to the discovery of Δ^3-carene by Simonsen (3) in 1920, we felt that a reinvestigation of the chemistry of turpentine of *P. ponderosa* was necessary.

The oleoresin was obtained, using conventional turpentining methods, from several vigorous trees about sixty or seventy years old, growing in the Sierra Nevada Mountains near Placerville, Calif., at an elevation of 2500 feet. Turpentine was distilled from a mixed sample of oleoresin under reduced pressure of 0.5 mm. Yield of turpentine was 15%.

Turpentine samples obtained from 28 individual trees were all levorotatory; the optical rotation $[\alpha]_D^{20}$ varied from $-39.1°$ to $-9.1°$. Refraction and density showed only minor variations.

Below are given the physical constants of two trees:

Tree	d_4^{25}	n_D^{20}	$[\alpha]_D^{20}$
No. 50	0.8572	1.4765	$-29°$
No. 60	0.8572	1.4760	$-10°$

Fractional Distillation of Turpentine.—The turpentine was distilled through an 80-cm. Vigreux-type column surrounded by a heating jacket and fitted with a partial reflux condenser. A reflux ratio of approximately 15:1 was employed. When the temperature had reached 172°, the 80-cm. column was replaced with a 20-cm. column of similar construction and the distillation was continued under reduced pressure. The quantities and physical constants of the fractions obtained are listed in Table I.

Identification of Components.—No α-pinene could be detected in the initial fractions of the sample under investigation.

Identification of *l*-β-Pinene in Fractions 4 and 6.—*l*-β-Pinene was identified by oxidation to nopinic acid by an alkaline solution of potassium permanganate. The procedure used was essentially a modification of that described by Dupont and Brus (4). The nopinic acid melted at 126°, when thoroughly dry. The reported melting point of nopinic acid is 126–127° (5).

These crystals showed no melting point depression when mixed with an authentic sample of nopinic acid. Both fractions 4 and 6 were found to contain *l*-β-pinene.

Identification of Δ^3-Carene in Fraction 9.—From fraction 9 a nitrosate was obtained following the procedure of Aschan (6). The nitrosate of Δ^3-carene is reported to melt at 142–146° (6), according to the rate of heating. The nitrosate of fraction 9 did not depress the melting point of an authentic sample of Δ^3-carene nitrosate. Therefore, we may assume that fraction 9 contains a considerable quantity of Δ^3-carene.

Identification of *dl*-Limonene in Fraction 13.—The physical constants of fractions 12 and 13 indicate that they consist largely of monocyclic terpenes with two double bonds. Fraction 12, probably because of the presence of isomeric terpenes, did not yield any crystalline derivatives, but fraction 13 gave a crystalline tetrabromide following the procedure of Wallach (7).

As is frequently the case when a mixture of *l*- and *dl*-limonenes is met, the tetrabromide finally obtained following recrystallization was *dl*-limonene-tetrabromide (dipentenetetrabromide) melting at 124–125° as it should (8). This gave no melting point depression with an authentic sample of *dl*-limonene tetrabromide.

Identification of *d*-Cadinene in Fraction 19.—According to the physical constants, fraction 19 consists largely of *d*-cadinene. This was confirmed by conversion of the fraction to its hydrochloride. The hydrochloride obtained melted at 117–118° and did not depress the melting point of a pure sample of *d*-cadinene dihydrochloride of m. p. 117–118° (9).

Attempt to Identify Fraction 23.—From Table II it is seen that fraction 23 consists mainly of a sesquiterpene. The combustion analysis on this oil after distillation over sodium showed a content of 87.78%

* Received October 24, 1949, from the U. S. Dept. of Agriculture Forest Service, Berkeley, Calif.

† Joint contribution from the Kerckhoff Laboratories of Biology, California Institute of Technology, and the California Forest and Range Experiment Station, which is maintained by the Forest Service, U. S. Dept. of Agriculture, in cooperation with the University of California.

TABLE I.—PHYSICAL CONSTANTS OF FRACTIONS FROM DISTILLATION OF *Pinus ponderosa* TURPENTINE

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Weight of Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	−161	742	0.2	0.848	....	.....
2	161–162	742	1.4	0.850	1.4701	−18.6°
3	162–163	742	1.9	0.850	1.4704	−17.8°
4	163–164	742	10.2	0.8678	1.4710	−17.0°
5	164–165	742	12.0	0.8684	1.4712	−15.4°
6	165–166	742	17.2	0.8568	1.4716	−14.5°
7	166–167	742	7.7	0.8660	1.4719	−12.3°
8	167–168	742	5.5	0.8614	1.4719	−11.9°
9	168–169	742	12.2	0.859	1.4719	−11.8°
10	169–170	742	1.4	0.850	1.4718	.....
11	170–171	742	2.6	0.853	1.4716	−12.2°
12	171–172	742	19.5	0.855	1.4712	−14.2°
				d_4^{25}		
13	85–89	30	2.0	0.859	1.4782	.....
14	89–99	30	0.3	0.871	1.4823	.....
15	99–109	30	0.2	0.892	1.4888	.....
16	109–119	30	0.5	0.919	1.4955	.....
17	119–129	30	0.4	0.930	1.4992	.....
18	129–139	30	0.3	0.930	1.4995	.....
19	139–149	30	1.3	0.923	1.4993	+16.7°
20	84–89	1	1.1	0.920	1.5002	+18.4°
21	89–99	1	0.4	0.919	1.5008	.....
22	99–109	1	0.6	0.917	1.5017	+17.8°
23	109–120	1	0.7	0.908	1.5012	+9.4°

carbon and 12.40% hydrogen. This analysis shows that the hydrocarbon is of the general formula $(C_5H_8)_n$.

The hydrochloride of the sesquiterpene was prepared. After three recrystallizations from glacial acetic acid, the final crystals consisted of colorless plates melting at 129–130°. None of the recorded sesquiterpene hydrochlorides melts at this temperature. The analysis and physical constants of fraction 23 and its trihydrochloride are collected in Table II and compared with a monocyclic and a bicyclic sesquiterpene.

From the molecular refraction data we have to conclude that we are dealing with a sesquiterpene with two double bonds, therefore containing two rings. From the hydrogenation data and the formation of a trihydrochloride it is seen that the sesquiterpene contains a labile ring system. In the sesquiterpenes we find a similar ring opening in the case of tricyclic copaene containing one double bond, which upon halogen acid addition is converted to a bicyclic dihydrochloride. The sesquiterpene of fraction 23 contains two double bonds and two rings, one of which is labile, and is converted to a trihydrochloride by opening one of the two ring systems.

From the above analyses it is seen that the turpentine contained approximately 51% *dl*- and *l*-β-pinene, 20% *dl*- and *l*-Δ^3-carene, 25% *l*-limonene and dipentene, 3% *dl*- and *d*-cadinene. In addition, we found a new sesquiterpene which we shall call *ponderene*. It is characterized by its trihydrochloride of m. p. 129–130°, present to the extent of about 0.5% of the turpentine. In apparent contradiction to the findings of Schorger, we could detect no α-pinene in the turpentine from *P. ponderosa*.

TABLE II.—CHARACTERISTICS OF HYDROCARBON IN FRACTION 23 OF *Pinus ponderosa* TURPENTINE

	Fraction 23		Cadinene[a]		Bisabolene[b]	
Boiling point	109–120°at 1 mm.		134–136° at 11 mm.		133–134° at 12 mm.	
Density	d_4^{25} = 0.9073		d_4^{20} = 0.9189		d_4^{25} = 0.8717	
Index of refraction	n_D^{25} = 1.5005		n_D^{20} = 1.5079		n_D^{25} = 1.4923	
Molecular refraction	M_D = 66.28 (found)		M_D = 66.10 (calcd. for 2 double bonds)		M_D = 67.8 (calcd. for 3 double bonds)	
Per cent carbon	87.78	found	88.23	calcd. for $C_{15}H_{24}$	88.23	calcd. for $C_{15}H_{24}$
Per cent hydrogen	12.40	found	11.77	calcd. for $C_{15}H_{24}$	11.77	calcd. for $C_{15}H_{24}$
Uptake of hydrogen per mole (micro-hydrogenation)	2.8 moles		2 moles		3 moles	
Molecular weight (micro rast)	216 (found)		204 (calcd.)		204 (calcd.)	
Hydrochloride melting point	129–130°		117–118°		79–80°	
Per cent carbon	57.90	found	74.81	calcd. for dihydrochloride	57.42	calcd. for trihydrochloride
Per cent hydrogen	8.69	found	10.46	calcd. for dihydrochloride	8.67	calcd. for trihydrochloride

[a] Henderson, G. G., and Robertson, A., *J. Chem. Soc.*, **125**, 1992(1924).
[b] Simonsen, J. L., "The Terpenes," Cambridge Univ. Press. Vol. II, 1932, p. 493.

PINUS BANKSIANA

Pinus banksiana, or jack pine, is the most northern American pine growing near the Arctic Circle in the MacKenzie River Valley. It extends as far south as northern New York and southeastern Minnesota. Oleoresin for the present work was obtained through the courtesy of the Lake States Forest Experiment Station, St. Paul, Minn. The oleoresin was collected from 35-year-old pines in the Chippewa National Forest, in the northern part of Minnesota, in the summer of 1946. Altogether about 7000 Gm. of oleoresin was obtained. It was semisolid, rosin acids being crystallized with ease, and contained a great deal of rain water. Upon distillation the oleoresin yielded 20.7% of turpentine, having the following properties: $d_4^{25°}$, 0.8595; n_D^{25}, 1.4670; $[\alpha]_D^{25}$, +9.75°.

The turpentine oil was fractionally distilled (Table III) and the presence of *dl*-α-pinene and small amount of *l*-α-pinene was shown by its physical constants and its conversion into the nitrosochloride with m. p. of 107–108°. The nitrosochloride showed no depression in melting point with a known sample of the compound. *l*-β-Pinene was identified by oxidation of fraction 7 with $KMnO_4$ and isolation of nopinic acid of m. p. 126–127°, showing no depression in melting point with an authentic sample of nopinic acid. From the data in Table III, it is estimated that the turpentine consists of 85% of *dl*- and *l*-α-pinene and 10% of *dl*- and *l*-β-pinene.

Distillation of the Oleoresin.—Four thousand grams of the oleoresin was distilled at a pressure of 0.01 mm. until the bath temperature reached 200°. There was obtained 1080 Gm. (27%) of the turpentine having the following properties: $d_4^{25°}$, 0.8552; $n_D^{27.5}$, 1.4637; $[\alpha]_D^{25}$, +0.91°.

Fractionation of the Turpentine.—Five hundred grams of turpentine was fractionated through a Vigreux column 40 cm. in length and equipped with a heating jacket and a partial reflux condenser. The relative quantities of the fractions, together with their physical properties, are listed in Table IV.

Following fractionation of the turpentine, the chief components of the turpentine were identified, both by reference to the physical properties of the individual fractions, and by conversion to appropriate solid derivatives.

Identification of *dl*-α-pinene.—The boiling range of fraction 2 strongly suggested that it was probably α-pinene, and its identity was confirmed by the preparation of the nitrolpiperidine derivative, m. p. 119–120°, following the method described by Wallach (10). No melting-point depression with an authentic specimen of *dl*-α-pinene nitrolpiperidine was observed. Microanalysis of the *dl*-α-pinene nitrolpiperidine gave the following results:

Anal.: Calcd. for $C_{16}H_{28}N_2O$: C, 72.68; H, 10.67; N, 10.60. Found: C, 72.65; H, 10.36; N, 10.83.

TABLE III.—PHYSICAL CONSTANTS OF FRACTIONS FROM DISTILLATION OF *Pinus banksiana* TURPENTINE

Fraction No.	Distillation Range: Temperature, °C	Distillation Range: Pressure, Mm.	Weight of Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	−156	740	0.8	....	....	.....
2	156–158	740	10.5	0.8608	1.4680	.....
3	158–159	740	66.8	0.8614	1.4730	−6.27°
4	159–160	740	4.3	0.8608	1.4734	−8.02°
5	160–161	740	1.4	....	....	.....
6	161–162	740	2.9	0.8632	1.4750	−10.66°
7	162–164	740	3.7	0.8620	1.4750	−11.68°
8	164–166	740	3.3	0.8637	1.4760	.....
9	166–168	740	1.4	0.8666	1.4768	−9.60°
10	168–170	740	0.8	0.8944	1.4814	.....
11	63–69	17	0.6	....	....	.....
12	Loss and residue	...	3.5	....	....	.....

PINUS CANARIENSIS

Pinus canariensis, Smith, is a native of the Canary Islands—it is frequently planted as an ornamental tree in warmer parts of the United States.

Inasmuch as *P. canariensis* is grouped together with *P. roxburghii* Sarg. (*P. longifolia* Roxb.) in the group *Longifoliae* subsection Parapinaster, section Diploxylon, it was felt that a chemical study of the turpentine of *P. canariensis* would be a valuable means of determining whether the two species were closely related biochemically as well as morphologically.

Oleoresin was obtained from twenty 25-year-old planted trees on the University of California campus, Berkeley.[1] Tapping was started July 17 and discontinued September 25, 1946. A little more than 4000 Gm. of semiliquid oleoresin was obtained.

Identification of *l*-Limonene.—The data in Table IV indicate the presence of *l*-limonene in fraction 11. Accordingly, the tetrabromide was prepared, using the procedure of Wallach (7). The tetrabromide melted at 124–125° and did not depress the melting point of an authentic sample of *dl*-limonene tetrabromide (8).

Anal.: Calcd. for $C_{10}H_{16}Br_4$: C, 26.34; H, 3.54. Found: C, 26.42; H, 3.73.

This result would indicate that fraction 11 contained much *dl*-limonene. However, the optical rotation is sufficiently high to indicate the presence of an appreciable quantity of *l*-limonene. Presumably, both isomers are present and the tetrabromide of the *dl*-isomer was obtained because of the greater solubility of the *l*-tetrabromide.

Identification of *d*-Bornyl Formate.—From a study of the quantities of the various fractions, it became apparent that yet another compound was

[1] In gathering and distilling the oleoresin, assistance was given by Professor Robert Cockrell, of the School of Forestry, University of California.

TABLE IV.—PHYSICAL CONSTANTS OF FRACTIONS FROM DISTILLATION OF *Pinus canariensis* TURPENTINE

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Weight of Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	100–155	742	1.0	0.854	1.4623	+2.93°
2	155–156	742	79.1	0.853	1.4637	+2.40°
3	156–157	742	6.5	0.853	1.4641	+0.06°
4	157–158	742	4.1	0.853	1.4648	−1.99°
5	158–159	742	2.3	0.852	1.4653	−4.09°
6	159–160	742	0.95	0.852	1.4660	−6.63°
7	160–161	742	0.68	0.852	1.4664	−7.22°
8	64–66	23	0.74	0.852	1.4672	−12.25°
9	66–75	23	0.59	0.849	1.4699	−27.14°
10	75–77	23	0.55	0.843	1.4720	−51.36°
11	77–81	23	0.37	0.842	1.4722	−60.99°
12	81–112	23	0.15	0.865	1.4752	−38.96°
13	112–116	23	0.07	0.923	1.4789	−6.48°
14	116–118	23	0.31	0.952	1.4782	+2.27°
15	118–122	23	0.10	0.954	1.4769	+4.32°
16	122–126	23	0.18	0.953	1.4762	+5.45°
17	126–129	23	0.19	0.945	1.4772	+7.66°
18	129–132	23	0.30	0.937	1.4800	+9.99°
19	132–139	23	0.21	0.928	1.4840	+13.23°
20	139–142	23	0.24	0.915	1.4902	+19.84°
	Loss and residue	...	1.3	...	...	...

present, chiefly in fraction 14. This fraction lay well within the boiling range of the terpene alcohols and esters. However, none of the usual tests indicated the presence of any alcohols.

When fraction 14 was saponified there was obtained in the steam distillate of the alcohol portion a solid substance having an odor like that of borneol. This solid sustance, when treated with 3,5-dinitrobenzoyl chloride in pyridine solution, gave a crystalline 3,5-dinitrobenzoate melting at 152–153°. *d*-Borneol 3,5-dinitrobenzoate is reported (11) to melt at 154°. The 3,5-dinitrobenzoate obtained from fraction 14 did not depress the melting point of an authentic specimen of *d*-borneol 3,5-dinitrobenzoate. After removal of the alcohol by steam distillation the presence of formic acid among the volatile acids could be demonstrated, indicating the ester present in fraction 14 consisted of *d*-bornyl formate. From the physical constants of the ester fractions the possibility of the presence of esters of higher acids is indicated.

The remaining 3% not accounted for consists of about ½% of an unidentified sesquiterpene, and a small amount of flask residue and distillation losses.

Since the turpentine of *P. longifolia* was found by Simonsen and Rau (12) to consist of 24.8% *l*-α-pinene, 37.6% dipentene and Δ³-carene, 9.7% β-pinene, and 20.3% sesquiterpene (longifolene), it appears that *P. canariensis* and *P. longifolia* are not related as closely physiologically as they are morphologically.

PINUS WASHOENSIS

Pinus washoensis is a newly discovered species (see Mason, H. L., and Stockwell, W. P., "A New Pine from Mount Rose, Nevada. Madrono 8," No. 2, 1945, pp. 61–63), growing at about 7000 feet near Mt. Rose, Washoe County, Nev. Apparently it is related closer to *P. ponderosa* than to any other pine growing in that locality, although it has certain characteristics (such as size and shape of cones) very different from those of *P. ponderosa*.

Oleoresin was collected from 19 young trees, probably fifty to sixty years old, by the members of the Institute of Forest Genetics during the summer of 1946. Weekly tapping started June 22 and was discontinued August 31. Altogether 12,854 Gm. of oleoresin having sugary consistency was collected. The samples of each tree were distilled separately (Table V). In average, content of turpentine in the oleoresin was 19.4%. Variations of the physical characteristics of the individual turpentine samples were found to be as follows: d_4^{25}, from 0.8522 to 0.8613; $n^{25}{}_D$, 1.4744 to 1.4777; $[\alpha]_D^{25}$, −9.75 to +8.40[2].

TABLE V.—VARIATION OF PHYSICAL CHARACTERS OF *Pinus washoensis* TURPENTINE OBTAINED FROM 19 DIFFERENT TREES

Tree No.	Density, d_4^{25}	Index of Refraction, $n^{25}{}_D$	Specific Rotation, $[\alpha]^{25}{}_D$
1	0.8603	1.4750	−1.60°
2	0.8597	1.4743	−6.61°
3	0.8597	1.4755	+0.97°
4	0.8577	1.4745	+8.10°
5	0.8603	1.4745	+10.15°
6	0.8567	1.4749	+4.42°
7	0.8544	1.4749	+6.78°
8	0.8597	1.4753	+1.74°
9	0.8599	1.4745	+7.95°
10	0.8522	1.4744	+7.94°
11	0.8590	1.4754	0.00°
12	0.8580	1.4757	−9.75°
13	0.8603	1.4777	+1.31°
14	0.8564	1.4757	+3.69°
15	0.8593	1.4757	+3.36°
16	0.8574	1.4768	+4.21°
17	0.8590	1.4768	+3.15°
18	0.8587	1.4768	+8.40°
19	0.8613	1.4770	+4.76°

[2] Three samples were levorotatory; one sample was optically inactive and 15 samples were dextrorotatory.

TABLE VI.—PHYSICAL CONSTANTS OF FRACTIONS FROM DISTILLATION OF *Pinus washoensis* (TREE 12) TURPENTINE

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Weight of Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	158–163	742	1.7	....	....	.....
2	163–164	742	3.2	....	1.4721	−6.55°
3	164–165	742	3.5	0.8586	1.4721	−9.06°
4	165–166	742	54.4	0.8557	1.4722	−8.76°
5	166–167	742	14.5	0.8551	1.4717	−8.65°
6	167–168	742	11.0	0.8660	1.4724	−7.56°
7	168–169	742	1.0	0.8685	1.4724	−7.81°
8	169–170	742	1.0	0.8592	1.4725	−7.92°
9	170–171	742	0.8	0.8627	1.4751	−10.51°
10	171–172	742	1.1	0.8598	1.4733	−15.64°
11	172–173	742	1.5	0.8592	1.4732	−15.70°
12	173–174	742	0.2	0.8592	1.4735	−15.45°
13	174–175	742	0.2	....	1.4736	.....
14	175–180	742	1.0	....	....	.....
15	100–130	17	1.0	0.9243	1.5044	.....
16	90–120	0.1	1.0	0.9293	1.5046	.....
	Residue	...	2.0	...	...	...

The fractionation data for trees 12 and 16 are represented in respective Tables VI and VII. While the data resemble each other closely, there is a distinct difference in behavior when tests were made for Δ^3-carene and β-pinene.

The oil from tree 12 formed a small quantity of Δ^3-carene nitrosate melting at 146–147°, when treated with anyl nitrite and nitric acid; while oil from tree 16 gave a copious precipitate of the nitrosate.

In tests for β-pinene it was found that tree 12 gave a good yield of nopinic acid, melting at 126°, while tree 16 gave none. The difference in optical activity of the turpentines from trees 12 and 16 is therefore caused by the presence of varying amounts of Δ^3-carene and the presence or absence of β-pinene. In both trees (12 and 16) α-pinene was found in the lower fractions; dipentene was present in the fractions boiling between 172–174° at 742 mm.

The physical constants of the high boiling distillates, namely, fractions 15 and 16 of tree 12 and fractions 12, 13, and 14 of tree 16 show the presence of cyclic sesquiterpenes.

SUMMARY

The oleoresin of *Pinus ponderosa* contains 15 per cent of volatile oil. The volatile oil consists of approximately 50 per cent *l*-β-pinene, 20 per cent *l*-Δ^3-carene, 25 per cent dipentene and *l*-limonene, 3 per cent *d*-cadinene and less than 1 per cent of a new sesquiterpene named ponderene which forms a trihydrochloride m.p. 129–130°.

The oleoresin of *P. banksiana* contains 21 per cent dextrorotatory turpentine. This turpentine consists of α-pinene (85 per cent) and β-pinene (10 per cent).

The oleoresin of *P. canariensis* contains 27 per cent volatile gum turpentine. This oil consists of approximately 94 per cent *dl*-α-pinene, 2 per cent limonene, and 1.5 per cent formic and other esters of borneol.

TABLE VII.—PHYSICAL CONSTANTS OF FRACTIONS FROM DISTILLATION OF *Pinus washoensis* (TREE 16) TURPENTINE

Fraction No.	Distillation Range: Temperature, °C.	Distillation Range: Pressure, Mm.	Weight of Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	150–161	742	0.6	0.8592	1.4707	.....
2	161–164	742	2.4	0.8550	1.4732	+3.74°
3	164–165	742	9.1	0.8557	1.4695	+4.15°
4	165–166	742	48.3	0.8598	1.4707	+8.90°
5	166–167	742	16.3	0.8580	1.4708	+8.51°
6	167–168	742	6.7	....	....	.....
7	168–169	742	1.8	0.8592	1.4710	+8.65°
8	169–171	742	1.6	0.8615	1.4725	+10.77°
9	171–173	742	1.6	0.8615	1.4728	+8.42°
10	173–175	742	3.0	....	1.4964	+8.36°
11	70–100	20	1.0	....	....	.....
12	100–120	20	1.1	0.9374	1.4988	+0.27°
13	80–110	0.1	0.4	....	1.5014	.....
14	110–130	0.1	1.0	....	1.5032	+6.75°
	Residue	...	5.0	...	...	...

The oleoresin of *P. washoensis* contains 18 to 22 per cent volatile oil. The volatile oil, obtained separately from several trees, consists largely of *d*-Δ^3-carene in varying quantities. In some trees with low carene content *l*-β-pinene is found. Small quantities of dipentene, α-pinene, and a cyclic sesquiterpene, are present in all trees examined.

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Hydrophilic Suppository Bases*

By W. C. WARD

The utility and versatility as suppository base materials of polyoxyalkylene derivatives of sorbitan partial fatty acid esters are described. These nontoxic, nonirritant, nonionic surface-active materials are particularly adaptable to suppository base formulation. They lend themselves readily to modification by various adjuvants producing desired melting characteristics. Incorporated medication is solubilized in many cases and readily released. A stable oil/water emulsion is formed with aqueous fluids. Rapidity of congealing is a further desirable feature of bases prepared with these materials.

MANY materials and combinations of materials have been utilized as suppository bases. Until recent years cocoa butter has been widely adopted and accepted as a base since it is solid at room temperature and liquid at body temperature. However, the shortcomings and annoying properties of this base have come to be recognized and investigations have been instituted to overcome these disadvantages. The fatty acid esters of the glycols have been studied by Bird (1) who developed a water-dispersible base consisting predominantly of propylene glycol monostearate.

Polymers of ethylene oxide have been proposed as bases which are water soluble and act by dissolution in body fluids rather than by melting.

The addition of emulsifying agents to cocoa butter was studied by Waxman and Eiler (2) and emulsifiable formulas produced. Lesser (3) has presented an excellent and thorough review of the many uses and types of suppository bases.

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EXPERIMENTAL

A series of polyoxyalkylene derivatives of sorbitan partial fatty acid esters, known by the trade name Tweens,[1] attracted our interest as possible ingredients of suppository bases. Investigational work showed promise and subsequent formulation demonstrated that this series possessed highly desirable features in formulating suppository bases.

The Tweens are nonionic surface-active materials, some of which are water soluble and others water dispersible. The lipophilic portion of the molecule is supplied by the ester group while the unreacted sugar hydroxyls and the polyoxyalkylene side chain furnish the hydrophilic or water orienting segments. There is presented, therefore, in this series a multiplicity of functional groups for emulsifying purposes which, by proper variance, affect the degree of water solubility from member to member. By the controlled addition of alkylene oxide units to the parent sorbitan partial fatty acid ester, derivatives ranging from solid, water dispersible to liquid, water soluble, and ultimately, if desired, solid, water-soluble types are produced. The transition from a solid, water-dispersible state to a solid water-soluble level encompasses derivatives of almost any desired melting point and degree of water solubility or dispersibility thus presenting a highly flexible and versatile series for use in suppository formulation.

[1] Atlas Powder Co.

Since suppositories exert their effect by melting at body temperature and/or dissolving in body fluids, an opportunity to achieve the dual action is presented in this versatile series. When specialized problems are met in compounding suppository bases, a series of derivatives possessing these variable physical properties is advantageous.

The present work has been concerned chiefly with the use of a polyoxyethylene derivative of sorbitan monostearate of the solid, water-dispersible type, Tween 61. This is a light amber-brown solid having the plastic and unctuous characteristics desirable in a suppository base. Tween 61 is insoluble in water, containing insufficient ethylene oxide units to produce water solubility, but it is dispersible in warm water and body fluids. Its melting range approximates body temperature and slight modification with suitable adjuvants readily produces a composition possessing desired melting features.

Tween 61 can be modified with other members of the Tween series to produce a more rapidly melting suppository base. A mixture of Tween 61 and Tween 60, a liquid water-soluble derivative, in the proper ratio produces a suppository base melting more rapidly than Tween 61. The addition of Tween 60 also confers some water-soluble character and more rapid emulsification upon the base. Glycol esters can be compounded with Tween 61 to prepare suitable supppository bases. A mixture of Tween 61 and glyceryl laurate[3] in a ratio of 85:15 makes an excellent low-melting suppository base. When the ratio is 90:10 the melting time is somewhat longer but the composition is more compatible with environmental temperature conditions. Sorbitan monoricinoleate and mineral oil are other adjuvants which can be used to produce acceptable bases.

To test the rate of melting of these compositions, a 2.5-Gm. suppository was placed in an incubator at 37.5° and the time noted at which liquefaction was complete. The preparation of Tween 61 (60%) and Tween 60 (40%) and that of Tween 61 (90%) and glyceryl laurate (10%) displayed a melting time of fifteen to seventeen minutes by this method. The combination of Tween 61 (85%) and glyceryl laurate (15%) exhibited a more rapid melting behavior of ten to thirteen minutes. Tween 61 itself, when tested by this procedure, showed a somewhat longer melting time.

Another test for melting which more nearly simulates actual conditions of use was the following: A 2.5- to 3.0-Gm. suppository was immersed in 6 cc. of water at 37–38° and the time for melting noted. By this procedure more rapid melting was observed in each case and it is assumed that the melting time in actual use would be less than that observed under either laboratory test.

The suppository bases prepared, using Tween 61 as the major component, melt to hydrophilic liquids which are self-emulsifiable in water, obviating the use of any surface-active agent of ionic character that might give rise to irritation of tissue. The surface-active properties of Tween 61 permit the ready formation of a stable emulsion with body fluids. The inherent property of the Tweens to effect the formation and maintenance of an extensive interface between disperse and continuous phases determines the stability of the emulsion. This stable emulsion spreads out smoothly like an ointment in the body cavity, effectively covering the area with a protective film. This effective coating action on mucosa is attributable to the surface-active properties of the Tweens. Further, a creamy emulsion of the o/w type favors longer lasting effects of incorporated medicaments due to a large contact area.

Bases prepared using Tween 61 have the property of solubilizing many medicaments. Phenylmercuric acetate tested for compatibility in these bases was found to be soluble at the level (1:2000) at which it was used. Boric acid was found to be soluble. The advantage of having medicaments completely soluble in a hydrophilic base is the increased availability to the mucous surfaces of both oil-soluble and water-soluble drugs since an adverse distribution coefficient to hinder or mask their action is avoided. It has been shown (4) that better antiseptic properties are obtained from phenylmercuric borate in o/w emulsions than in the w/o type. Excellent antibacterial activity from these bases, under the conditions of the test employed, is displayed by phenylmercuric acetate. A clear zone of inhibition of 7 mm. is produced when tested by the agar cup plate method of the U. S. Food and Drug Administration (4) using *Staphylococcus aureus* as the test organism.

Suppository bases of the Tween type have the unique and desirable property not found in other bases of congealing very rapidly to a firm wax at ordinary temperatures. This simplifies manufacturing technique and, because of this rapidly congealing characteristic, the deleterious effects of hot climates can be readily overcome by simply cooling the preparation in water.

SUMMARY

The Tweens, particularly Tween 61, offer a number of advantages when used as, or in, suppository bases. Tween 61 possesses a melting point that is readily modified by small amounts of suitable adjuvants to any desired range. Dispersibility in fluids due to its surface-active properties covers mucous membranes with a smooth protective coating, produces greater retention of the molded body, and a minimal tendency to leak. The formation of an emulsion of the o/w type also allows ready diffusion of medicament. The bases prepared with Tweens are nontoxic, nonirritating in use, contain no volatile or odorous substance, not excessively greasy, neutral in reaction and stable. No impairment of melting behavior is noted over a twelve-month period. There is no tendency toward rancidity and most medicaments are compatible with these bases. The bases possess an elasticity and pliability which affords ease in use.

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[3] Glyco Products Co.

Action of Sulfonamides upon Bacterial Dehydrogenases*

By MICHELE GERUNDO

By means of the methylene blue reduction test, the action of sulfonamides upon the dehydrogenase system was studied. No effect by sulfonamides was produced on glucose dehydrogenase but a strongly inhibitory action by them was produced on lactic dehydrogenase. From the evidence accumulated it is believed that bacteria which do not possess succinic dehydrogenase are more susceptible to Sulfasuxidine and probably to other sulfonamides. Among the bacteria capable of dehydrogenating succinic acid in the presence of Sulfasuxidine are *Shigella sonnei*, *Proteus vulgaris*, *Escherichia coli*, and *Aerobacter aerogenes*.

THE RELATIONSHIP of sulfonamides to dehydrogenases is perhaps the most important aspect of sulfonamide action if the true bacteriostatic mechanism is that of blocking cellular respiration.

Sevag and Shelburne (1–3) advanced the view that sulfonamides block bacterial respiration, in the absence of, as well as during, growth, by formation of drug-protein-coenzyme complex.

West and Coburn (4) suggested that sulfapyridine and nicotinic acid compete for the same position in the coenzyme molecule and that the bacteria are unable to form the coenzyme necessary for their dehydrogenases in presence of sulfapyridine. Barron and Jacobs (5) found that Prontylin had an inhibiting effect upon lactate oxidation and McLeod (6) found that sulfapyridine-fast strain of Pneumococcus I is unable to dehydrogenate glycerol, lactate, or pyruvate.

In contrast to this view of the mechanism of action of sulfonamides, there are several reports that the system of oxidases is blocked by sulfonamides (7–10); however, this second mechanism cannot apply to anaerobic organisms.

There are various respiratory mechanisms by means of which bacterial cells may oxidize a substrate. One important system may be the Szent-Gyorgyi cycle, which is considered as a catalytic system of H carriers between foodstuffs and cytochrome (11). All the paths outside the cytochrome-hemin ferment cycle are called accessory respiratory systems (12).

The purpose of this investigation was to establish the mechanism of action of succinylsulfathiazole upon enteric bacteria and to find, if possible, the reasons for the differences in susceptibility of the various microorganisms (13).

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EXPERIMENTAL

The study of dehydrogenases was carried out in Thunberg tubes under the conditions given in the table headings. The tubes were evacuated by means of a manifold with the use of a hyvac. At the beginning, "resting cells" were prepared according to Quastel's technique (14), but after preliminary tests and on the basis of work carried out by Hirsch (15) and Henry (16), cultures forty-eight hours old were centrifuged, washed only once and then resuspended in phosphate buffer solution. Strains used in these experiments had been freshly isolated from patients at Los Angeles County Hospital, except for *Pseudomonas aeruginosa* and *Proteus* OX 19 which had been obtained from the American Type Culture Collection. For preparing bacterial emulsions, a Lumetron photoelectric colorimeter was used to obtain the same density in all the experiments for any single species. The forty-eight-hour cultures were chosen because they show the maximum dehydrogenase activity and no active division.

RESULTS

Succinylsulfathiazole (Sulfasuxidine) in a concentration of 1:500 delayed or inhibited reduction of methylene blue by bacteria suspended in glucose phosphate buffer solution (Table I). It is noteworthy that *Salmonella schottmuelleri*, *Eberthella typhosa*, *Escherichia coli*, fairly sulfonamide-resistant microorganisms, showed almost no delay in methylene blue reduction in the presence of sulfonamide, whereas *Escherichia coli* var. *communior*, *Alkaligenes faecalis*, *Shigella dysenteriae*, *Shigella alkalesens*, and *Staphylococcus aureus* did not reduce or reduced very little methylene blue.

Table II shows the results obtained by adding coenzyme I to the methylene blue test. The reduction time of methylene blue was not much changed, except for *Staphylococcus aureus* 12, which showed no reduction in the tube containing glucose-sulfathiazole. Generally, the strains which are sulfonamide-susceptible showed little, if any, reduction of methylene blue. Reduction was not very evident in most of the coenzyme-sulfathiazole tubes. In testing succinic acid dehydrogenase (Table II), it seems that the activity varied with the bacterial

TABLE I.—EFFECT OF SULFASUXIDINE ON GLUCOSE DEHYDROGENASE[a,b]

Organism	Reduction of Methylene Blue: With Sulfasuxidine 30 Min.	With Sulfasuxidine 1 Hr.	Without Sulfasuxidine 30 Min.	Without Sulfasuxidine 1 Hr.	Buffer
Salmonella schottmuelleri	...	Complete	...	Complete	No change
Shigella paradysenteriae	...	Complete	...	Complete	No change
Escherichia coli 10	...	Complete	...	Complete	No change
Staphylococcus aureus 12	...	Incomplete	...	Incomplete	No change
Alkaligenes faecalis I	...	No change	...	No change	No change
Escherichia coli var. *communior* 15	x ...	Incomplete	Complete	...	No change
Shigella alkalescens	...	0	...	Incomplete	No change
Shigella dysenteriae	...	Incomplete	...	Incomplete	No change
Eberthella typhosa	...	Complete	...	Complete	No change
Shigella sonnei	Complete	...	Complete	...	No change
Escherichia coli 229S	...	Incomplete	...	Complete	No change
Salmonella abortusovis	...	Partial	...	Partial	No change

[a] Buffer pH 7.4, 2 ml.; glucose 5%, 1 ml.; Sulfasuxidine 1:500, 5 ml.; methylene blue 1:2000, 1 ml.; bacterial emulsion, 48 hr. old, 1 ml.
Control contains saline instead of Sulfasuxidine.
[b] Active cells used in the test. Results obtained with "resting cells" are very similar (see references 14, 15, and 16).

TABLE II.—EFFECT OF SULFATHIAZOLE UPON GLUCOSE DEHYDROGENASE AND OF BACTERIA UPON SUCCINIC ACID AND SUCCINYLSULFATHIAZOLE[a]

Organism	Reduction of Methylene Blue: Glucose	Glucose Coenzyme I	Glucose Sulfathiazole	Glucose Coenzyme Sulfathiazole	Coenzyme Sulfathiazole	Sulfasuxidine	Succinic Acid	Buffer
Proteus vulgaris	x	x	x	x	x	xxx	xx	...
	xxx	xxx	xxx	xxx	...			
Aerobacter aerogenes	xx	xx	xx	xx	x	0	xxx	...
	xxx	xxx	xx	xxx	...			
Pseudomonas aeruginosa	x	x	x	x	x	0	x	...
	xx	xx	xx	x	xx			
Salmonella schottmuelleri	xx	xx	x	x	x	xx	xx	...
	xxx	xxx	xxx	xxx	xx			
Shigella paradysenteriae	xx	xx	xx	xx	...	...	...	...
	xxx	xxx	xx	...	...			
Escherichia coli 10	x	xx	xx	xx	xx	0	xxx	...
	xxx	xxx	xxx	xxx	...			
Staphylococcus aureus 12	...	...	...	xx	...	0	x	...
	x	x	...	x	...			
Alkaligenes faecalis I	...	xx	xx	xxx	x	x	x	...
	xx	xx	xx	xx	xx			
Escherichia coli var. *communior* 15	x	x	xx	xx	...	...	...	...
	x	xxx	x	xx	...			
Proteus vulgaris 0X 19	x	xx	xx	xx	x	0	x	...
	xxx	xxx	xxx	xxx	...			
Salmonella paratyphis	xx	...	...	...	...	0	0	...
	xx	xx	xx	xx	x			
Shigella alkalescens	xx	xx	xx	xx	...	...	...	...
	xx	xx	xx	xx	x			
Streptococcus faecalis	...	x	...	...	...	0	x	...
Shigella dysenteriae	x	x	...	...	...	0	x	...
	x	x	x	x	...			
Proteus vulgaris Su 35	xx	xx	xx	xx	xx	...	...	...
	xxx	xxx	xx	xxx	...			

[a] 0 = no decol., x = partial, xx = incomplete, xxx = complete, x = slightly less than x.
Phosphate buffer pH 7.4, 2 ml.; methylene blue 1:5000, 1 ml.; (glucose 0.5%), 0.5 ml.; (sulfathiazole 1:2000), 0.5 ml.; (coenzyme I 1:2000), 0.1 ml.; (succinic acid 1%), 1 ml.; (Sulfasuxidine 1:1000), 2 ml.; active bacterial suspension, 1 ml.; saline, 2 ml.
For each strain the top line is the reading four hours after mixing, the bottom line twelve hours after mixing, except for the columns of Sulfasuxidine and succinic acid which give sixty-minute readings.

species. *Proteus vulgaris* showed more prompt reduction, but *Aerobacter aerogenes* and *Escherichia coli* showed more intense reduction. The strains which had been found susceptible to Sulfasuxidine showed either very slight or no succinic dehydrogenase activity.

An experiment carried out with active bacteria by adding Sulfasuxidine as a substrate to the buffer solution indicated that *sulfonamide-resistant* strains were able to reduce methylene blue, whereas *susceptible* strains gave practically no reduction. These results (Table III) are rather difficult to interpret, as one is not sure whether the succinic acid moiety or the whole succinylsulfathiazole molecule is being dehydrogenated. On the basis of this experiment, considering that only the more resistant strains

TABLE III.—EFFECT OF SULFATHIAZOLE ON REDUCTION OF METHYLENE BLUE WITH SUCCINATE AND FUMARATE[a]

Organism	Reduction of Methylene Blue					
	Succinic Acid	Sulfathiazole	Succinic Acid Sulfathiazole	Fumaric Acid	Fumaric Acid Sulfathiazole	Buffer
Proteus vulgaris	xx	x	xx	xxx	xxx	...
	xx	xxx	xxx			
Aerobacter aerogenes	x	x	x	...	...	...
	xxx	xx	xxx			
Pseudomonas aeruginosa	...	x	...	...	...	...
Salmonella schottmuelleri	x	x	...	...	x	...
	xxx	xx				
Shigella paradysenteriae	...	x	...	...	...	...
	x	...	...			
Escherichia coli 10	x	x	...	...	...	...
	xxx	xx	xxx	...	xx	
Staphylococcus aureus	...	...	...	...	...	...
Alkaligenes faecalis I	x	xx	x	x	...	...
	xx	xxx	xx	xxx	xxx	
Escherichia coli var. *communior* 15	...	x	...	...	x	...
	xx	xx	xx	...	x	
Salmonella paratyphis	...	...	x	xxx	...	...
	x	xx	xx	...		
Shigella alkalescens	...	...	...	...	...	...
	xx	x	xx			
Streptococcus faecalis	...	...	...	...	...	...
Shigella dysenteriae	...	x	...	...	x	...
	x					
Shigella sonnei	x	...	...	...	...	...
	...	...	...	...	...	...
Brucella suis	...	...	...	...	...	...
Proteus vulgaris X 19	...	x	...	...	...	...
	xx	xxx	x	...		
Eberthella typhosa	xxx	x	xx	x	...	...
	...	xx	...	xxx	xxx	
Proteus vulgaris Su 33	...	...	...	...	x	...
Alkaligenes faecalis 110	...	...	...	...	x	...
Salmonella sp.	...	...	...	...	...	...

[a] Phosphate buffer pH 7.4, 2 ml.; methylene blue 1: 5000, 1 ml.; (succinic acid 1%), 0.5 ml.; (sulfathiazole 1: 2000), 0.5 ml.; (*para*-amino-benzoic acid 1: 1000), 0.5 ml.; (fumaric acid 1%), 1 ml.; (vitamin B_1, 1: 1000), 1 ml.; active bacterial suspension 1 ml.; saline, 2 ml.

Cultures thirty-six hours old used in these tests.

In parentheses—the additions made as indicated in the table. Degree of reduction indicated as in Table II.

NOTE: The tubes containing succinic acid, sulfathiazole and *para*-amino-benzoic acid showed results similar to those containing succinic acid sulfathiazole; therefore the results have been omitted.

The tubes containing vitamin B_1 and fumaric acid showed results similar to those containing fumaric acid; therefore the results have been omitted.

TABLE IV.—EFFECT OF SULFATHIAZOLE ON LACTATE AND ACETATE DEHYDROGENASES[a]

Organism	Reduction of Methylene Blue					
	Sodium Lactate		Sodium Lactate Sulfathiazole		Sodium Acetate	Sodium Acetate Sulfathiazole
	30 Min.	1 Hr.	30 Min.	1 Hr.	1 Hr.	1 Hr.
Proteus vulgaris	x	xx	x	xx	xx	...
Pseudomonas aeruginosa	...	...	...	...	...	...
Salmonella schottmuelleri	x	xxx	...	x	...	...
Shigella paradysenteriae	x	x	x	x	...	...
Escherichia coli 10	xxx	xxx	x	xx	...	...
Staphylococcus aureus	x	x	...	...	...	...
Escherichia coli var. *communior* 15	x	xxx	...	x	x	...
Salmonella paratyphis	...	...	...	...	...	...
Shigella alkalescens	...	xxx	...	xx	...	...
Streptococcus faecalis	...	...	...	...	...	...
Shigella dysenteriae	...	...	...	...	...	...
Shigella sonnei	...	...	...	...	...	...
Brucella suis	...	...	...	...	xx	...
Proteus vulgaris X 19	xx	xxx	xx	xxx	xx	...
Eberthella typhosa	x	xx	...	xx	...	...
Alkaligenes faecalis 110	...	...	...	...	x	...
Brucella abortus	...	...	...	...	...	...

[a] Phosphate buffer pH 7.4, 2 ml.; methylene blue 1: 5000, 1 ml.; (sodium lactate 5%), 0.5 ml.; (sodium acetate 5%), 0.5 ml.; (sulfathiazole 1: 2000), 1 ml.; bacterial emulsion, 1 ml.; saline, 2 ml.

Cultures were forty-eight hours old.

In parentheses—additions made only as indicated in the table.

showed reduction, one might be inclined to believe that the sulfonamide was being oxidized by these bacteria to an inactive compound, whereas those strains which did not possess such mechanism were inhibited by the drug. For this reason, sulfathiazole and succinic acid were used in the following experiment (Table III). In the presence of sulfathiazole alone, the resistant bacteria developed a certain degree of reduction of the dye, whereas the more susceptible strains showed only slight or no reduction. In the tubes containing succinic acid plus sulfathiazole, the reduction of methylene blue was strongly inhibited except for sulfonamide-resistant strains. *Para*-aminobenzoic acid did not modify this reaction to any great extent. Likewise, coenzyme I added to succinic acid-sulfathiazole did not modify the results, although Potter (17) claims that it may take part in the succinate cycle. When fumaric acid was used as a substrate (Table III), only a few bacteria showed some reduction of the dye. Vitamin B_1 added to the substrate had very slight influence upon the reaction.

No reduction of the dye was evident when a combination of succinic and fumaric acids was used as a substrate. Fumaric acid is known to serve as an acceptor of H as follows:

$$\text{Succinic acid} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \text{Fumaric acid}$$

The lactate dehydrogenase appeared to be strongly influenced by sulfathiazole (Table IV). Here, too, those bacteria which were relatively more resistant to the drug showed strong dehydrogenase activity when no sulfathiazole was present. The susceptible bacteria which demonstrated either a complete inhibition or a very delayed reaction presented a weak reaction in the absence of sulfathiazole. This may point to a correlation between activity of the dehydrogenase system and susceptibility to sulfonamides. Lactate dehydrogenase is strongly influenced by sulfathiazole. Even resistant bacteria, like *Salmonella schottmuelleri* and *Eberthella typhosa*, showed definite but short delay in reduction in the presence of the drug. With sodium acetate as a substrate (Table IV), the bacteria which are able to reduce methylene blue were inhibited in the tube containing sulfathiazole.

DISCUSSION

The experimental findings are mostly in agreement with those of other workers in the field. When the system of dehydrogenases is blocked, bacteria which can act upon succinate and use the succinic acid cycle as a link between dehydrogenases and cytochrome may escape inhibition by sulfonamides. As the principal respiratory mechanism is blocked, this accessory path may assume a predominant role.

Thiamine was used, because its action must be linked with that of cocarboxylase (18); however, in this experiment, its presence did not alter the results.

Glucose dehydrogenase is probably the least affected of all the series although its action may be delayed in many instances when the concentration of sulfonamide is high. Its existence as an entity has been questioned by Potter (19). Sevag and Gots (20) believe that methylene blue may mask the inhibition of pneumococcic glucose dehydrogenase by sulfonamides.

In these experiments, by using sulfonamide as a substrate in buffered solutions, the more resistant bacteria were able to reduce methylene blue as if the drug were being oxidized by these bacteria. While it is possible that some organisms oxidize sulfonamide and may not be susceptible to it, it is more likely that the oxidized form of sulfonamide is the inactive one and that in its presence the bacteria may grow without interference. Now, in order to form a drug-protein-coenzyme complex, it is more logical to suppose that the drug must be in a reduced form and therefore unable to accept H from the substrates. If we admit the formation of an oxidation product, then we could reasonably accept the idea of reversible oxidation-reduction and, therefore, of a sulfonamide carrying on the function of a coenzyme.

Those bacteria which are able to use the Nebenatmung path of the citric acid cycle may sidetrack the action of sulfonamide and thus escape its inhibition mechanism, as pointed out earlier; however, this statement cannot be applied universally. For example, *Eberthella typhosa*, which is not susceptible to the action of sulfonamide, is unable to utilize succinic acid.

A number of bacteria possess systems which may act as accessory catalyst by producing a series of coupled oxidation-reduction between split products of foodstuffs through the mediation of suitable electroactive mediators (Szent-Gyorgyi accessory catalysts). We could reasonably suppose that bacteria lacking these accessory catalysts are perhaps more susceptible to sulfonamide than those provided with them. As the action of sulfonamide blocks the principal dehydrogenases and the utilization of pyruvate, the accessory catalysts become more predominant and functional in the continuation of bacterial metabolism. The chick embryo, for example, which does not utilize succinic acid, is unable to withstand injection of succinate into the yolk sac (21).

While this explanation is hypothetical, the experimental results so far obtained point to its probability. It seems reasonable to assume that the principal action of sulfonamides is upon the dehydrogenases system and not on some theoretical essential metabolite.

SUMMARY

The action of sulfonamides upon the dehydrogenase system was studied by means of the methylene blue reduction test. Glucose dehydrogenase is not affected by sulfonamides, but lactic dehydrogenase is strongly inhibited by them. It seems that bacteria which do not possess succinic dehydrogenase are more susceptible to Sulfasuxidine and probably to other sulfonamides. Among the bacteria capable of dehydrogenating succinic acid in the presence of Sulfasuxidine are *Shigella sonnei*, *Proteus vulgaris*, *Escherichia coli*, and *Aerobacter aerogenes*.

In conclusion, these experiments tend to prove: (*a*) ability of the naturally resistant strains to reduce methylene blue using sulfathiazole as a substrate, whereas susceptible strains were unable to

do so; (*b*) postulated susceptibility to sulfonamides of cells which lack ability to dehydrogenate succinic acid and resistance to these drugs when cells by-pass dehydrogenase inhibition by utilizing citric acid cycle via succinate; and (*c*) inhibition of respiration by sulfonamides.

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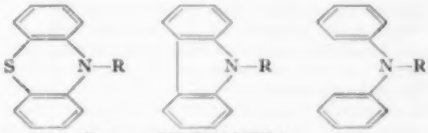
Antihistaminics: The N-*Beta*-Dimethylaminoethyl Derivatives of Carbazole and Diphenylamine*

By J. H. BURCKHALTER, VERLIN C. STEPHENS†, and LUTHER A. R. HALL

Although β-dimethylaminoethyl chloride hydrochloride is a very important intermediate which has been especially useful in the synthesis of various antihistaminic agents, publications mentioning its use often neglect to refer to a convenient method of preparation. A simplified laboratory procedure for the preparation of this intermediate is reported and a description is given of its use in the synthesis of two antihistamine-type compounds derived from carbazole and diphenylamine.

FOR MANY years β-dialkylaminoethyl bromide hydrobromides have been known. However, the standard method of preparation requires the use of large volumes of hydrobromic acid (1). The less expensive analogous chlorides are preferred since their preparation is simpler and their reactivity is sufficient for the synthesis of antihistaminic types (2). Ordinarily β-dialkylaminoalkyl chloride hydrochlorides are prepared in good yields by treatment of β-dialkylaminoalkanols with an excess of thionyl chloride in chloroform or benzene (3). A recent article on the German commercial preparation of Atabrine describes the action of thionyl chloride on β-diethylaminoethanol hydrochloride without solvent (4).

Our variation of the method for the preparation of β-dimethylaminoethyl chloride has several advantages over those previously given. Therefore, we believe that a detailed description of the process is warranted. The usual steps employed include either distillation of the solvent or of excess thionyl chloride (3). In our procedure the slight excess of thionyl chloride is removed by reaction with the recrystallizing solvent to give only gaseous by-products (ethyl chloride, sulfur dioxide, and hydrogen chloride). Since no solvent and only a slight excess of thionyl chloride are used, the process is more economical. This is especially true, for in the simplified process one less operation is involved. The yields (91 to 94 per cent) obtained are as good or better than any previously obtained by other investigators.



(I) (II) (III)

Interest in phenothiazine antihistaminics such as 3015RP (I) and its methyl derivative, 3277RP (5), suggested the analogous carbazole (II) and

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diphenylamine (III) derivatives as compounds of possible value. Following the general preparative procedure of Eisleb (6), who had synthesized the diethyl homolog of II as a possible antispasmodic, the sodium salt of carbazole or diphenylamine was made by use of sodamide. To the sodium salt of each was added an equivalent amount of β-dimethylaminoethyl chloride. The free bases of the desired products were distilled and the hydrochlorides readily prepared therefrom.

After the completion of our laboratory studies, 9-(β-dimethylaminoethyl)-carbazole (II) was reported by others to possess only slight antihistamine activity (7). The product has been mentioned elsewhere (8), but no physical constants are available. The hydrochloride of N-(β-dimethylaminoethyl)-diphenylamine (III) has also been reported, but no pharmacodynamic properties are given (9).

The two products were tested as antihistamine agents by Dr. Graham Chen and Mr. C. R. Ensor of Parke, Davis & Co., Detroit, Michigan. 9-(β-Dimethylaminoethyl)-carbazole hydrochloride (II) was ineffective in guinea pigs when administered intraperitoneally in doses of 25 mg./Kg., whereas N-(β-dimethylaminoethyl)-diphenylamine hydrochloride (III) given subcutaneously was about one-tenth as active as diphenylhydramine hydrochloride (Benadryl).

EXPERIMENTAL

β-Dimethylaminoethyl Chloride Hydrochloride.—Thionyl chloride (290 Gm., 2.44 moles) [Note 1] was placed in a dry one-liter flask fitted with a sealed mechanical stirrer, an efficient reflux condenser, and a 500-ml. dropping funnel. The reaction flask was cooled in an ice bath throughout the entire period of operation as the reaction was very exothermic. β-Dimethylaminoethanol (210 Gm., 2.25 moles) [Note 2] was added dropwise through the funnel to the cooled thionyl chloride [Note 3] over a period of an hour with the accompanying copious evolution of sulfur dioxide [Note 4].

After all the dimethylaminoethanol had been added, the ice bath was removed and the reaction mixture stirred for an hour [Note 5]. The temperature of the mixture was 35–50°. The reaction mixture was a brown semisolid slush of desired product together with a slight excess of thionyl chloride.

The entire contents of the reaction flask was transferred to a two-liter beaker containing approximately a liter of absolute alcohol [Note 6]. The resulting brown solution was heated to boiling on a hot plate during which time there was a copious evolution of the gases named in Note 6. The solution was filtered hot leaving a small amount of insoluble material. After cooling the filtrate, the desired product was obtained as beautiful white crystals, which were collected on a Buchner funnel and dried in a vacuum desiccator over phosphorus pentoxide [Note 7]. The yield of pure product melting at 201.5–203° was 227–272 Gm. (70–84%).

Upon evaporation of the last filtrate second-crop crystals of high purity, white color, and first-crop melting point were obtained; 33–69 Gm. (10–21%). Total yield 296–305 Gm. (91–94%).

Notes.—1. Eastman practical grade is satisfactory.

2. The commercial grade, Eastman or Carbide and Carbon, of dimethylaminoethanol is satisfactory.

3. Continued and efficient cooling of the reaction vessel is needed to prevent too vigorous an evolution of sulfur dioxide and a subsequent loss of thionyl chloride through a trapping of this reagent by effluent gases. Cooling also prevents too high a reaction temperature. The reaction proceeds more smoothly if the temperature is kept below 50°.

4. Care should be taken to see that the dropping funnel inlet does not become clogged with solid product. The reaction must be carried out in an efficient hood or with a suitable trap in order to remove the noxious sulfur dioxide formed.

5. The reaction mixture may be stirred for a longer time and allowed to stand overnight without affecting the yield.

6. The alcohol not only converts the excess thionyl chloride to gaseous by-products (sulfur dioxide, hydrogen chloride, and ethyl chloride), but it also serves as the recrystallizing solvent for the desired product.

7. The product is somewhat hygroscopic, especially in humid weather. It should be dried in a vacuum desiccator to prevent the formation of hydrated forms.

9-(β-Dimethylaminoethyl)-carbazole Hydrochloride II.—A mixture of 16.7 Gm. (0.1 mole) of carbazole, 4.7 Gm. (0.12 mole) of sodamide (10), and 100 ml. of dry benzene was stirred and heated at refluxing temperature for two hours for the formation of the sodium salt of carbazole. To the stirred mixture a benzene solution of β-dimethylaminoethyl chloride free base, prepared from 7 Gm. (0.12 mole) of the hydrochloride,[1] was added. Stirring and refluxing were continued for eight hours. The mixture was cooled and the solid sodium chloride was separated by filtration. Benzene was removed by distillation, and the residue was then distilled under reduced pressure; 14.5 Gm. (60% yield) of yellow oil boiled at 190–210° (4–6 mm.). An excess of alcoholic hydrogen chloride added to the free base yielded a white crystalline hydrochloride. Recrystallized from alcohol it melted at 240–242° (dec.).

Anal.—Calcd. for $C_{16}H_{18}N_2 \cdot HCl$: Cl, 12.90. Found: Cl, 13.14.

N-(β-Dimethylaminoethyl)-diphenylamine Hydrochloride (III).—The desired product was prepared by the foregoing procedure using 35 Gm. (0.2 mole) of diphenylamine, 10 Gm. (0.25 mole) of sodamide, and 30 Gm. (0.21 mole) of β-dimethylaminoethyl chloride. The product distilled as the free base at 160–175° (6 mm.). Yield 40 Gm. or

[1] In the conversion to the free base the amount of water was kept at a minimum. Concentrated sodium hydroxide was added, and the mixture extracted with three different portions of benzene. The benzene extract of the amine was dried well over anhydrous potassium carbonate. For isolation of the free base without the use of solvent, see Burtner, R. R., *J. Am. Chem. Soc.*, **71**, 2578(1949).

80%. The hydrochloride recrystallized from alchol melted at 250–253° (dec.).[8]

Anal.—Calcd. for $C_{16}H_{20}N_2 \cdot HCl$: Cl, 12.81. Found: Cl, 12.85.

SUMMARY

A simplified and convenient laboratory procedure for the preparation of β-dimethylaminoethyl chloride hydrochloride has been described in detail.

This intermediate has been used in the synthesis of two antihistamine-type compounds derived from carbazole and diphenylamine. The latter derivative was found to be about one-tenth as active as Benadryl.

[8] Carrara, *et al.*, found 252–254°; reference 9.

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Identification of Sulfa Drugs by Refractive Index Measurements at Their Melting Points*

By J. H. BAIRD and H. A. FREDIANI

The necessary physical data have been determined for differentiating between sulfadiazine, sulfaguanidine, sulfamerazine, sulfanilamide, sulfathiazole, and sulfapyridine by making use of their melting points and the refractive indices of the melts.

THE determination of two physical constants of a relatively pure chemical substance usually serves adequately for qualitative identification. Two such constants that may be readily determined on solids (1) are melting point and refractive index of the melt. Under the conditions described below it is also possible to determine the dispersion and the temperature coefficient of the refractive index. These additional properties may be used to confirm the identity of questionable samples.

Keenan (3) has recently reported on the optical crystallographic data of some crystalline sulfonamides. The use of these crystalline properties serves well for their microscopic identification. To supplement these data, our laboratory has studied the refractive indices of the melts as a more rapid, although not as elegant, technique.

* Received October 5, 1949, from the Chemical Control Division, Merck & Co., Inc., Rahway, N. J.

APPARATUS

A Fisher refractometer supplied with heater head, transformer capable of varying the voltage between 0 and 60 volts, and a thermometer with a range of 20 to 300° was employed for the simultaneous measurements (2).

The numerical values reported herein have been obtained with the Fisher apparatus since it permits a more rapid determination with less time consumption than the technique suggested by the Koflers (4).

PROCEDURE

A crystal or two of the substance to be investigated is placed in the well formed between the prism and the heater head of the refractometer. The temperature is rapidly brought to within 5 or 10° of the melting point expected and then slowly raised at the rate of 1 or 2° per minute until the sample actually melts. This may be readily ascertained since at the melting point the virtual image appears on the scale of the refractometer. The temperature at which this virtual image makes its appearance is noted and the refractive index read. If desired, the temperature is changed by manipulation of the transformer so that a sufficiently large difference in refractive index and temperature is effected in order to yield values from which temperature coefficients may be calculated.

RESULTS

Table I includes the melting points obtained by this technique, the refractive indices of the melts measured, the literature values for melting points, and the refractive indices previously reported (4).

TABLE I

Compound	Literature Melting Point	Observed Melting Point	Refractive Index of Melt — Determined	Refractive Index of Melt — From Koflers' Data (4)
Sulfadiazine[a]	255–256	254–256	1.585	Compound not listed
Sulfaguanidine	189–190	191–192	1.610	Compound not listed
			1.617 @ 160°	
			1.628 @ 120°	
Sulfamerazine[a]	234–238	236–237	1.568	Between 1.550–1.561
Sulfanilamide	165–166	163–164	1.588	1.586
Sulfathiazole[a]	201–202	200–201	1.639	Between 1.635–1.648
Sulfapyridine	191–192	191–193	1.610	1.609
			1.625 @ 160°	
			1.640 @ 120°	

[a] Sulfadiazine, sulfamerazine, and sulfathiazole begin to char after melting.

Of the six sulfa drugs listed, four have characteristic melting points and refractive indices so that no difficulty is encountered in their differentiation. For sulfaguanidine and sulfapyridine, however, both the melting points and refractive indices are sufficiently similar so that definite differentiation or positive identification of either of these would be impossible. Both of these compounds may be supercooled quite extensively. By slowly permitting the refractometer heater head to cool, it is possible to measure the refractive index of each of these compounds at 160 and 120°. As may be noted from the preceding table, the temperature coefficient for sulfapyridine is sufficiently greater so that measurement of the refractive index of the supercooled liquid at 120 or 160° suffices to differentiate it from sulfaguanidine.

DISCUSSION

Four of these sulfa compounds, namely sulfamerazine, sulfanilamide, sulfathiazole, and sulfapyridine, have been studied by the Koflers on a microscope hot stage. Their technique, although precise and reproducible, involves a more tedious procedure. Their method is to melt the substance to be identified on a hot stage under the microscope and to add to this melt fragments of glass of known refractive index. The glass chosen should have a refractive index closely approximating that of the melt. This involves selection by trial and error from a special set of glass refractive index standards. After a glass is found that closely approximates that of the melt, the temperature of the melt is changed until the refractive index of the melt and of the glass are identical. This is noted by use of the Becke line technique. By use of this technique, values for the index of refraction of a specific sample may be determined to ±0.0001. Unless one measures the temperature coefficient and interpolates, the refractive index measured is that of the melt at some temperature above the melting point of the actual sample. Since it is impossible to raise the temperature until it matches that of the glass fragments for substances that melt with decomposition (i.e., sulfathiazole), it is only possible to indicate a refractive index range. As a typical example, the Koflers have reported that for sulfathiazole the closest approximation they could estimate was that the refractive index of the melt was between 1.6482 and 1.6346. By the use of the Fisher refractometer, a reproducible value of 1.639 may be readily obtained.

Since all of the samples mentioned above had similar dispersion, i.e., between 0.015 and 0.020 refractive index units between the red and green images, it was impossible to use this property for their differentiation.

CONCLUSION

It has been found that simultaneous measurements of melting point and refractive index at the melting point serve to differentiate sufficiently four of the six sulfa drugs studied. Sulfaguanidine and sulfapyridine may be distinguished from the other four, but not from each other because of the similarity in their melting points and refractive indices. Since both of these compounds may be readily supercooled to as much as 70° below the measured melting point, the temperature coefficients for these compounds have been investigated and found to suffice for their differentiation.

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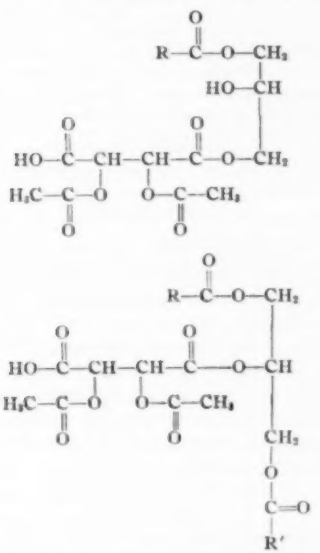
Correlation between the Acute Toxicity and the Rate of Elimination of Tartaric Acid and Certain of Its Esters*

By THEODORE L. SOURKES and THEODORE KOPPANYI

A report is presented on the results of a comparative study of the acute toxicity and the rate of renal excretion of tartaric acid following administration of tartaric acid, diacetyl tartaric acid, and diacetyl tartaric acid esters of mixed mono- and diglycerides obtained from natural fats. The toxicity of the first two compounds has been correlated with their high rate of renal excretion. The mixed mono- and diglycerides have been found to be well absorbed from the intestinal tract, to show no acute toxicity in large doses, and to result in the available tartaric acid being slowly excreted by the kidney.

THE METABOLISM of tartaric acid and its salts has been the subject of many investigations in the past, some of the most extensive being those of Underhill and co-workers (1, 2). Recently a new group of esters of tartaric acid has become available for use as emulsifying agents especially, but not exclusively, in the food industry. These compounds are the diacetyl tartaric acid esters of mixed mono- and diglycerides obtained from natural fats (3). These esters, hereinafter referred to as TEM, must comprise several types of molecular species, two of which are illustrated in the following representations:



where R and R′ represent the hydrocarbon portion of the fatty acid chains.

In the course of a study on the metabolism of TEM it was observed that while large amounts of tartaric acid or its diacetyl derivative are acutely toxic to dogs, correspondingly large doses of TEM are without toxicity. Correlated with this are the observations that following administration to dogs of nontoxic doses of tartaric acid or diacetyl tartaric acid (DTA), the rate of urinary excretion of tartaric acid is very high, but this rate is relatively low when TEM is given, even though it is efficiently absorbed from the intestinal tract.

EXPERIMENTAL

Chemical.—The fate of the compounds studied was followed in the metabolism experiments by using the tartaric acid portion as a "tracer." Tartaric acid was determined as follows: an appropriate aliquot of the sample to be analyzed was made up to 20 ml. with water, 2 Gm. of KOH were added, and the mixture was refluxed for two hours. After washing down the condenser with water, the digest was acidified with sufficient HCl (1:1) and 2 Gm. of Norit were added. The mixture was boiled for ten minutes and filtered. If the filtrate was milky it was poured back through the same paper until clear. An aliquot of the clarified filtrate was analyzed for tartaric acid by a modification of the visual colorimetric method of Underhill and associates (4): to 10 ml. of the tartaric acid solution, add 1 ml. of glacial acetic acid followed by 4 ml. of 5% sodium metavanadate solution (aqueous). After standing for fifteen minutes at room temperature the tubes are examined in the photoelectric colorimeter at 520 mμ (or a green filter may be used). A blank is used which contains 10 ml. of water instead of the tartaric acid solution. The color follows Beer's law over a range of concentrations up to about 30 mg. % but a calibration curve extends the useful range.

The color is given by *d*- and *dl*-tartaric acid, but not by the *meso*-compound (4, 5). The present authors have checked this and have also compared the behavior of a sample of *l*-tartaric acid (Brickman and Co., Montreal). The two optically active forms and the racemic mixture are colorimetrically equivalent. Underhill (4) found that lactic acid in high concentrations interferes. We have tested an additional number of compounds and have found that the following give no color over the blank:

* Received November 3, 1949, from the Department of Pharmacology and Materia Medica, Georgetown University School of Medicine, Washington 7, D. C.

TABLE I.—CUMULATIVE PER CENT RENAL EXCRETION OF TARTARIC ACID BY DOGS FOLLOWING ADMINISTRATION OF TARTARIC ACID, DIACETYL TARTARIC ACID, AND TEM

	Dog No.							
	1	2	3	4	5	6	7	8
Weight, Kg.	14.7	5.4	4.4	3.9	8.1	5.4	6.8	3.4
Substance adm.[a]	TA	DTA	DTA	DTA	TEM	TEM	TEM	TEM
Amount adm., Gm.	7.35	3.89	3.17	2.80	23.14	14.56	18.33	9.17
Urine at end of:								
4th hr.	13.8	19.0	4.7	13.0	6.5	5.9	1.8	2.4
8th hr.	37.7	47.8	25.2	29.5	9.0	8.7	13.9	4.7
12th hr.	48.9	58.6	31.8	36.5	11.2	...	18.8	6.7

[a] TA = tartaric acid; DTA = diacetyl tartaric acid.

Diacetyl tartaric acid, diethyl tartrate, and TEM; formic, oxalic, malic, fumaric, and citric acids; alanine, serine, threonine, isoleucine, tyrosine, histidine, and methionine; formaldehyde and acetaldehyde; glucose; and urea. Cysteine and ascorbic acid both reduce the vanadium to the bluish green V3 + state, but cystine and dehydroascorbic acid have no effect on the reagent. After standing at room temperature for many hours, the citric-metavanadic acid solution shows some evidence of reduction (slight blue color). From the above it is evident that besides the optically specific requirements, the hydroxyl groups and carboxyl groups of tartaric acid must be free to achieve a positive test.

Biological.—Acute toxicity was studied in dogs. The compound to be tested was administered by stomach tube as a solution or slurry in water. It was observed that whereas the animals did not survive following administrations of a large dose of tartaric acid (5 Gm./Kg. body weight) or of the diacetyl derivative (7.2 Gm./Kg.) there were no untoward effects after TEM was given in comparable amounts, based on its tartaric acid content (27 Gm./Kg.). Of the six dogs receiving 27 Gm./Kg. of TEM, two were sacrificed ten days following administration. The microscopic study of their kidneys showed no evidence of nephrotic changes characteristic of tartaric acid damage.

To study the rate of elimination of tartaric acid in the urine, lightly anesthetized (nembutalized) dogs were administered relatively small doses of the test compound by duodenal tube. The dose given was 0.5 Gm./Kg. body weight of tartaric acid or its equivalent in available tartaric acid from the diacetyl derivative (0.72 Gm./Kg.) or TEM (2.72 Gm./Kg.). Urine was collected by catheter at four-hour intervals until the end of the twelfth hour, and the tartaric acid content following alkaline hydrolysis of each sample was determined as described in the "chemical" section. The results are shown in Table I.

The data indicate that in twelve hours, under the experimental conditions, about half of the tartaric acid administered as such is excreted in the urine and that diacetyl tartaric acid behaves similarly. On the other hand, dogs receiving TEM, excrete less than 20% of the available tartaric acid in the same time by way of the kidney. Indeed, the rate of excretion of tartaric acid following TEM administration is only about 25% to 30% of that observed following either tartaric acid itself or its diacetyl derivative. That this slower rate for TEM is not due to lack of absorption or delayed absorption from the intestinal tract was indicated in the following way: at the end of the twelfth hour, the intestinal contents of dogs No. 7 and No. 8 were collected, mixed in the Waring Blendor, evaporated to dryness on the steam bath, and exhaustively extracted with ether, in which TEM is soluble. The ether-soluble residue was weighed and found to be equivalent to 12.6% and 11.4%, respectively, of the weight of TEM administered. If this residue consisted of TEM alone, then 88% of the TEM administered could be accounted for by intestinal absorption. The digestibility coefficient of TEM, as determined in the adult rat is 95% (6).

DISCUSSION

By comparing the results of the acute toxicity and renal excretion studies above, it would appear that tartaric acid is absorbed from the intestinal tract rapidly enough to reach toxic concentration in the body fluids and tissues. The same is true of diacetyl tartaric acid, although it is, of course, not known whether this compound itself is toxic or only after its hydrolysis in the intestinal tract or tissues to liberate tartaric acid. The lack of toxicity of TEM under comparable conditions may be due to slower hydrolysis, so that an effective toxic concentration of tartaric acid is never reached. Chronic toxicity studies of TEM are in progress.

SUMMARY

A comparative study has been made of the acute toxicity and the rate of renal excretion of tartaric acid, following administration of tartaric acid, diacetyl tartaric acid, and TEM. The toxicity of the first two compounds is correlated with their high rate of renal excretion. On the other hand, TEM, which is well absorbed from the intestinal tract, shows no acute toxicity in large doses and its available tartaric acid is but slowly excreted by the kidney.

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The Colorimetric Determination of Various Antihistamine Compounds*

By F. J. BANDELIN, E. D. SLIFER, and R. E. PANKRATZ

Various antihistamine compounds react with ammonium reineckate reagent in aqueous solution producing precipitates which can be isolated, dissolved in acetone, and determined colorimetrically by measuring the transmittance at 525 mμ. A general method for antihistamines is given and a procedure for their extraction from tablets, capsules, and ointments, prior to determination. A procedure for the separation of methapyrilene hydrochloride from a mixture of acetylsalicylic acid, acetophenetidin, and caffeine is given. Determination on a number of commercial preparations gave results well within the tolerance limits for such preparations. Results were reproducible with variations generally within one per cent.

SINCE THE initial studies of Bovet and Staub (1), literally hundreds of antihistamine compounds have been synthesized and studied pharmacologically. Although these compounds have attained tremendous pharmaceutical and clinical importance, little has appeared concerning chemical methods for their quantitative determination in pharmaceutical preparations. Gelvin, McGavack, and Drekter (2) utilized a modification of the Brodie test (3) for organic bases and reported on the change of concentration of these bases in blood, urine, and spinal fluid of patients receiving diphenhydramine hydrochloride (Benadryl) and tripelennamine hydrochloride (Pyribenzamine). Perlman (4) applied the cyanogen bromide test to compounds containing a pyridine nucleus and utilized the fluorescence produced by the reaction as a means of quantitative measurement. In searching for a method applicable to various antihistamine compounds in pharamceutical products we investigated ammonium reineckate as an analytical reagent and found, in preliminary studies, that most of the commercially available compounds were quantitatively precipitated from aqueous solution by this reagent. The insoluble reineckate could be dissolved in acetone and measured colorimetrically or spectrometrically.

Reagents

1% Sulfuric Acid.

Acetone.

Ammonium Reineckate Reagent: 1.0 Gm. of ammonium reineckate is shaken with 50 ml. of distilled water for ten minutes, then filtered through a hard filter paper. This solution should be prepared fresh daily.

Wash Solution.—Two milliliters of ammonium reineckate reagent in sufficient distilled water to make 1000 ml.

* Received November 28, 1949, from the Research Laboratories of Flint, Eaton and Co., Decatur, Ill.

Standard Solution of the Antihistamine Compound.—Two hundred milligrams of the compound, accurately weighed, is dissolved in 100 ml. of distilled water in a volumetric flask. This solution contains 2 mg. of the compound per ml. Solutions containing 2 mg. per ml. of each of the following compounds were prepared for investigation:

Methapyrilene hydrochloride[1], tripelennamine hydrochloride[2], doxylamine succinate[3], pyranisamine maleate,[4] diphenhydramine hydrochloride,[5] prophenpyridamine maleate,[6] thenzylamine hydrochloride,[7] phenindamine tartrate,[8] chlorothen hydrochloride,[9] antistine hydrochloride.[10]

Phenindamine tartrate and antistine hydrochloride required standard solutions containing 5 mg. of compound per ml.

PROCEDURE

A solution containing from 2 to 10 mg. of the antihistamine compound in 10 ml. of 1% H_2SO_4 is pipetted into a 20-ml. beaker. The beaker is placed in a shallow ice bath and 5 ml. of ammonium reineckate reagent is added dropwise from a pipette while swirling the contents of the beaker. The beaker is then allowed to stand in the ice bath for one hour at the end of which time the precipitate is collected by filtering through a 15-ml. sintered glass crucible of medium porosity with the aid of gentle suction. The precipitate in the crucible is washed with two 5-ml. portions of cold wash water (5°) after which the

[1] Supplied by Abbott Laboratories as Thenylene Hydrochloride; by Eli Lilly and Co. as Histadyl; and by Flint, Eaton and Co. as Pyrathyn Hydrochloride.
[2] Supplied by Ciba Pharmaceutical Products, Inc., as Pyribenzamine Hydrochloride.
[3] Supplied by Wm. S. Merrell and Co. as Decapryn Succinate.
[4] Supplied by Merck and Co. as Neo-Antergan Maleate.
[5] Supplied by Parke, Davis & Co. as Benadryl Hydrochloride.
[6] Supplied by Schering Corp. as Trimeton Maleate.
[7] Supplied by Wyeth, Inc., as Neo-Hetramine Hydrochloride.
[8] Supplied by Hoffmann-La Roche, Inc., as Thephorin Tartrate.
[9] Supplied by Lederle Laboratories as Tagathen Hydrochloride.
[10] Supplied by Ciba Pharmaceutical Products, Inc.

suction is continued to draw any excess water from the precipitate. The precipitate is then dissolved in acetone by placing the crucible in a 65-mm. glass funnel whose stem has been drawn out into a capillary having a diameter of about 2 mm. This is done so that the stem of the funnel will fit into the neck of a 25-ml. volumetric flask when the funnel is supported so that the end of the capillary is just above the mark on the neck of the volumetric flask. Five milliliters of acetone is pipetted into the crucible so as to wash down the sides with the solvent. A one-hole rubber stopper fitting just inside the top is placed in the crucible and a glass tube equipped with a rubber bulb is inserted just through the hole in the stopper. Gentle air pressure is applied to the surface of the acetone by compressing the bulb with the hand, thus forcing the acetone through the funnel carrying the precipitate in solution with it into the volumetric flask. This operation is repeated several times until all the precipitate is dissolved and the solution in the flask is made up to volume with acetone. The solution is well shaken and transmittance is determined at 525 mμ in a suitable photoelectric colorimeter or spectrophotometer.

PREPARATION OF THE STANDARD CURVE

Pipette 1, 2, 3, 4, and 5 ml. of the standard solution of the antihistamine compound into each of five 20-ml. beakers and adjust the volume of each to 10 ml. with 1% H_2SO_4. Precipitate the antihistamine compound as the reineckate as described in the procedure. Transmittance of the acetone solutions is determined at 525 mμ and the values obtained plotted against concentration on semilog paper. Results for the compounds were essentially straight lines in conformity with Beer's law.

The standard curve for methapyrilene hydrochloride is given as an example in Fig. 1. Other antihistaminic compounds investigated gave similar results with some variation of the slope of the standard line peculiar to and consistent with that particular compound.

REPRODUCIBILITY OF RESULTS

Reproducible colorimeter or spectrophotometer results were obtained with all the various compounds listed and consistent readings were obtained at various concentration levels with repeated determinations.

Using diphenhydramine hydrochloride (Benadryl) as an example, results were obtained on ten separate determinations at the 6.0-mg. level at 525 mμ as shown in Table I.

Similar results at different levels of concentration with variations of the same magnitude were obtained with all the other compounds investigated.

TABLE I

Detn.	Transmittance, %	Detn.	Transmittance, %
1	68.0	6	68.0
2	68.0	7	68.0
3	68.5	8	68.0
4	68.0	9	68.2
5	67.8	10	68.2

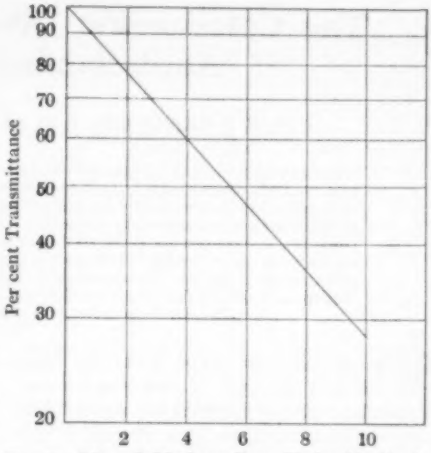

Fig. 1.—Mg. of Methapyrilene Hydrochloride in 10 ml.

PROCEDURE FOR TABLETS OR CAPSULES

Crush 20 tablets in a mortar and reduce to a fine powder, or, in the case of capsules, remove the contents of 20 capsules and mix well in a mortar. Weigh an aliquot equivalent to 50 mg. of the antihistamine compound and transfer it quantitatively to a 100-ml. beaker and digest on a steam bath with 50 ml. of 1% H_2SO_4 for thirty minutes. At the end of this time, cool and filter through paper into a 100-ml. volumetric flask and make up to volume by adding 1% H_2SO_4 through the filter. A 10-ml. aliquot of this solution, accurately measured, is precipitated with ammonium reineckate reagent as described in the procedure. Measure the transmittance of the acetone solution of the reineckate at 525 mμ and determine the amount of antihistamine compound by reference to the standard curve for the particular compound under determination.

METHOD FOR OINTMENTS

Weigh a sample of the ointment equivalent to 50 mg. of the antihistamine compound and dissolve or suspend in 50 ml. of petroleum ether in a separatory funnel. Extract the petroleum ether phase by shaking it with four 10-ml. portions of 1% H_2SO_4. Collect the combined acid extracts in a second separatory funnel and shake them with 5 ml. of petroleum ether. Filter the acid solution through a funnel containing a pledget of cotton moistened with 1% H_2SO_4 into a 50-ml. volumetric flask. Wash the second separatory funnel with several small portions (2 to 3 ml.) of 1% H_2SO_4 and add through the funnel to make the volume 50 ml. Pipette a 10-ml. aliquot of this solution into a 20-ml. beaker and precipitate with 5 ml. of ammonium reineckate reagent as described in the procedure. Determine the transmittance of the acetone solution of the reineckate at 525 mμ and calculate the amount of antihistamine compound by reference to the standard curve for the particular compound under determination.

A number of the above-listed commercial products in the form of tablets or capsules were assayed for the antihistamine compound content. Values consistent with the stated potency were obtained as indicated in Table II.

TABLE II

Product	Stated Potency, Mg.	Found Potency, Mg.
Product A, 50-mg. capsules	50	51.4
Product B, 50-mg. tablets	50	50.4
Product C, 50-mg. tablets	50	50.1
Product D, 50-mg. capsules	50	50.4
Product E, 50-mg. tablets	50	49.8

Since these compounds generally are depressants and cause soporific reactions in many individuals, it is likely that stimulants such as the sympathomimetic amines might be administered concurrently in the same preparation to counteract depression. With this in mind we investigated the determination of the various antihistamines in the presence of ephedrine, amphetamine, and desoxyephedrine. It was found that in a mixture with each of these compounds up to quantities equivalent to those of the antihistamine compound, no interference resulted.

Combinations of antihistamine compounds with various antipyretic compounds have found wide application in the treatment of the common cold and because of the pharmaceutical importance of such mixtures, we studied the separation and determination of methapyrilene hydrochloride in combination with the usual A. P. C. formula containing acetylsalicylic acid 3.5 gr., acetophenetidin 2.5 gr., caffeine 0.5 gr., and methapyrilene hydrochloride, 0.33 gr. The following scheme based on the separation method of Holt (5) for acetylsalicylic acid, acetophenetidin, and caffeine was found applicable and expedient.

PROCEDURE

Grind 20 tablets in a mortar and reduce to a fine powder. Suspend an accurately weighed aliquot (about 1.5 Gm.) in 25 ml. of 4% hydrochloric acid in a separatory funnel. Extract with one 40-ml. and four 20-ml. portions of ether. The collected ether extracts are washed in a second separatory funnel with 10 ml. of 4% hydrochloric acid and 10 ml. of water and these are washed through two additional 20-ml. portions of ether. The water is added to the original solution which is retained for the determination of caffeine and methapyrilene hydrochloride. The collected ether extracts containing the acetylsalicylic acid and acetophenetidin are washed with one 25-ml. portion of 5% sodium bicarbonate solution and one 15-ml. portion of water. These aqueous extracts are separately washed through two 20-ml. portions of ether and the combined aqueous phase used for the determination of acetylsalicylic acid. The last two 20-ml. portions of ether are added to the ether solution and the combined ether extracts are used for the determination of acetophenetidin.

The original aqueous phase containing the caffeine and methapyrilene hydrochloride is extracted with four 30-ml. portions of chloroform, each of which is consecutively washed through a 5-ml. portion of 5% hydrochloric acid. The combined chloroform extracts are used for the determination of caffeine.

The aqueous solutions are collected in a 100-ml. volumetric flask. A 10-ml. aliquot is pipetted into a 20-ml. beaker and the methapyrilene hydrochloride determined as heretofore described in the procedure for the antihistamine compounds. Transmittance of the acetone solution of the methapyrilene reineckate was determined at 525 mμ and the concentration determined by reference to the standard curve.

DISCUSSION

Certain tertiary amines and quaternary ammonium salts produce precipitates with ammonium reineckate. In the preceding method, this principle has been applied to the antihistamine compounds and adapted as an analytical method. Other similar compounds, including the alkaloids, would interfere with the determination if present in combination with the antihistamine compounds.

The range of concentration required for determinations is such that dilutions may be readily prepared without excessive dilution and the accompanying dilution error.

Determinations carried out on solutions containing definite and uniform increments of the pure compound yielded values for each compound which, when plotted on semilog paper, yielded a straight line in conformity with Beer's law. Results were reproducible with variations generally within 1%.

Commercial preparations assayed gave results well within the tolerance limits for such preparations without difficulty or interference from the excipient materials.

The sympathomimetic amines investigated produced no precipitate with the ammonium reineckate reagent in concentrations up to 100 mg. of compound per 10-cc. aliquot, either separately or in the presence of a similar concentration of the antihistamine compound.

In the separation from acetylsalicyclic acid, acetophenetidin, and caffeine, methapyrilene hydrochloride was the only compound studied. Other similar compounds could undoubtedly be handled in the same manner giving satisfactory results.

The method is simple, rapid, and expedient and has the advantage of being applicable to all the common antihistamine compounds investigated. Although a method for the colorimetric determination of various antihistamine compounds was described (6) while this paper was in preparation, this method is limited to compounds containing a pyridyl group, since it is dependent upon the Konig reaction (7) utilizing cyanogen bromide and aniline.

SUMMARY

A method for the colorimetric determination of antihistamine compounds is described. The method is dependent upon the precipitation of the antihistamine compounds from aqueous solution with ammonium reineckate. The reineckate is dissolved in acetone and transmittance determined at 525 mμ on a photoelectric colorimeter or spectrophotometer. Concentration is determined by reference to a standard curve.

The method has been tried with success on a number of commercially available antihistamine compounds, both in pure form and in the usual dosage forms such as tablets, capsules, and ointments. Procedures for determination of the compounds in those dosage forms are given.

The presence of various sympathomimetic amines such as ephedrine, amphetamine, and desoxyephedrine did not interfere with the determination.

A method for the separation and determination of methapyrilene hydrochloride from a mixture of acetylsalicylic acid, acetophenetidin, and caffeine is given.

The method is rapid, simple, and expedient, giving reproducible results with a variation generally within one per cent.

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The Photofluorometric Estimation of Estrogens*

By GORDON A. GROVES and MERVYN J. HUSTON†

A satisfactory photofluorometric method for the estimation of certain crystalline estrogens is described. This method was successfully adapted to the assay of commercial preparations of estrone in oil, aqueous suspensions of estrone, and estradiol tablets.

IN THE Kober (1) colorimetric assay of estrone it has been noted that a green fluorescence is produced when sulfuric acid is added to estrone. This fluorescent phenomenon was used qualitatively by Sala (2) as a test for pregnancy, and by Cuboni (3) to determine the presence of estrone in the urine of pregnant mares. More recently several procedures have appeared in the literature (4–7) for the quantitative estimation of estrogens by means of fluorescence. It was the purpose of this investigation to develop a sensitive photofluorometric method for the assay of commercial pharmaceutical preparations of estrogens.

EXPERIMENTAL

The intensity of fluorescence was determined by means of a Coleman photofluorometer, Model 12. In the technique described by Jailer (5, 6) the filter combination B-2, PC-9A was employed; for the method of Bates and Cohen (7) and Finklestein, *et al.* (4) a B-1, PC-1 combination was used. Filter B-2 transmits at 436 mμ and filter B-1 at 365 mμ.

Attempts to reproduce the results obtained by Jailer (5, 6) were unsuccessful. Concentrations used by him developed intensities beyond the range of our galvanometer and lower concentrations did not give a linear relationship between intensity of fluorescence and amount of estrone.

Finklestein, *et al.* (4) reported a method using phosphoric acid to produce fluorescence of the estrogens. A comparison of sulfuric and phosphoric acids indicated that the former developed a much higher degree of fluorescence.

Using a B-1, PC-1 filter combination, Bates and Cohen (7) were able to show a linear relationship between intensity of fluorescence and concentration of estrone over a range of 1 to 10 μg. We obtained satisfactory results with this procedure but found that increased sensitivity and accuracy could be obtained by using a higher reaction temperature.

PROCEDURE

The procedure employed by us was as follows: An aliquot of an alcoholic solution of the estrogen was placed in the cuvette and to this was added 1 ml. of 95% sulfuric acid. The contents of the tube were mixed and heated in a boiling water bath for ten minutes. The tube was removed, cooled, and 8 ml. of 65% sulfuric acid added. The tube was again cooled and the intensity of fluorescence measured in the photofluorometer.

A "blank" was run with all determinations. This "blank" solution was prepared by combining in the same manner and amounts all the ingredients with the exception of the estrogen being investigated.

Adjustment of the lamp intensity, and thus instrument sensitivity, was accomplished by means of

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a standard reference solution. The use of such a standard produced fewer variabilities in the readings obtained. For the purpose of a standard reference we employed a solution containing 10 μg. of quinine sulfate per milliliter of 0.1 *N* sulfuric acid. The galvanometer was adjusted to give a reading of 80 with this solution. This adjustment was made immediately preceding each determination.

RESULTS

Results with Crystalline Hormones

In Table I are presented the galvanometer readings obtained with estrone, estradiol, estriol, and estradiol benzoate. Aliquots of a solution containing 1 mg. of the estrogen per 100 ml. of 95% ethanol were employed for test purposes.

The results are presented graphically in Fig. 1. The degree of fluorescence exhibited by equal quantities of the estrogens decreases in the following order: estrone, estradiol benzoate, estradiol, and estriol.

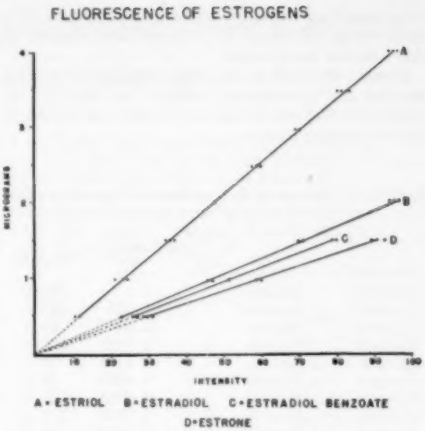

Figure 1

Results with Pharmaceutical Preparations of Estrogens

The application of the above procedure to commercial preparations requires preliminary extraction and purification. The assay results reported were obtained with the procedures described below. In the assay of some commercial preparations more frequent extractions may be necessary for complete removal of the estrogens. This method of assay has not been successfully applied to preparations containing other than one pure hormone.

1. Solutions of Estrone in Oil.—One milliliter of the oil solution of estrone was measured into a 125-ml. separatory funnel containing 25 ml. of petroleum ether. Ten milliliters of 2 *N* sodium hydroxide was added, the mixture was shaken vigorously for two minutes, and the two layers were allowed to separate completely. The aqueous layer was transferred to a second 125-ml. separatory funnel. The extraction was repeated with two additional 10-ml. portions of 2 *N* sodium hydroxide solution, each of which was added to the second funnel. The petroleum ether solution was discarded. A 50% *v/v* solution of sulfuric acid was added to the alkaline solutions until a permanent opalescence formed (acid to litmus). The mixture was cooled thoroughly and 25 ml. of anesthetic ether was added. The mixture was shaken thoroughly for one minute and the two layers were allowed to separate. The acid layer was transferred to a second 125-ml. separatory funnel and the extraction was repeated with another 25 ml. of anesthetic ether. The acid layer was discarded. The ether layer was washed with two, 5-ml. portions of 5% sodium carbonate solution and then with two 5-ml. portions of distilled water (8). The ether solution was transferred to a 50-ml. beaker and evaporated to a volume of 3 or 4 ml. The final few milliliters of liquid were removed by directing a stream of

TABLE I.—CALIBRATION OF THE PHOTOFLUOROMETER FOR ESTROGENS

	μg.	Galvanometer Readings					
Estrone	0.5	30.0	30.0	30.0	30.0	29.0	31.0
	1.0	61.0	62.0	61.0	61.0	62.0	61.0
	1.5	91.0	93.0	91.0	91.0	90.0	91.0
	2.0	100+[a]	100+	100+	100+	...	...
Estradiol	0.5	24.0	24.0	24.0	24.0	24.0	24.0
	1.0	48.0	49.0	49.0	48.0	48.0	48.0
	1.5	71.0	72.0	72.0	72.0	72.0	72.0
	2.0	96.0	94.0	96.0	96.0	94.0	95.0
	2.5	100+	100+	100+	100+	...	...
Estriol	0.5	12.0	11.0	11.0	12.0	12.0	12.0
	1.0	24.0	24.0	22.0	24.0	24.0	25.0
	1.5	36.0	37.0	36.0	36.0	35.0	36.0
	2.0	48.0	48.0	48.0	48.0	48.0	48.0
	2.5	60.0	60.0	61.0	59.0	60.0	60.0
	3.0	72.0	71.0	72.0	72.0	72.0	71.0
	3.5	84.0	82.0	84.0	84.0	83.0	85.0
	4.0	96.0	95.0	97.0	96.0	96.0	97.0
Estradiol Benzoate	0.5	26.0	26.0	26.0	27.0	26.0	26.0
	1.0	52.0	51.0	51.0	52.0	52.0	53.0
	1.5	78.0	78.0	78.0	79.0	79.0	78.0
	2.0	100+	100+	100+	100+	...	...

[a] 100+—Reading beyond galvanometer range.

compressed air over the surface. The residue was dissolved in 100 ml. of 95% ethanol and aliquots of this solution were tested.

Results obtained in assaying a number of control samples and commercial ampuls are recorded in Table II. The results agree well with the known or stated concentrations.

TABLE II.—ASSAY OF SOLUTIONS OF ESTRONE IN OIL

Preparation	Vehicle (Oil)	Known or Stated Concn., Mg.	Determined Concn., Mg.	Recovery, %
Control	Sesame	1	1	100.0
Control	Sesame	1	1.005	100.5
Control	Peanut	1	0.9910	99.1
Control	Peanut	1	1.005	100.5
Control	Sesame	0.2	0.1978	98.9
Control	Sesame	0.2	0.1984	99.2
Control	Peanut	0.2	0.2028	101.4
Control	Peanut	0.2	0.2046	102.3
Control	Sesame	0.1	0.0983	98.3
Control	Sesame	0.1	0.1009	100.9
Control	Peanut	0.1	0.100	100.0
Control	Peanut	0.1	0.1016	101.6
Company A	Sesame	1	1.009	100.9
Company A	Sesame	1	1.031	103.1
Company A	Peanut	1	1.000	100.0
Company A	Peanut	1	1.003	100.3
Company B	Peanut	1	0.991	99.1
Company B	Peanut	1	0.988	98.8
Company A	Peanut	0.2	0.200	100.0
Company A	Peanut	0.2	0.1992	99.6
Company A	Sesame	0 1	0.1016	101.6
Company A	Sesame	0.1	0.1003	100.3

2. Estrone Aqueous Suspensions.—One milliliter of the solution of estrone was diluted to 10 ml. with distilled water to provide a more convenient working volume. This solution was extracted with two 25-ml. volumes of anesthetic ether. The ether extracts were combined and backwashed with two 5-ml. volumes of distilled water. The ether solution was evaporated to dryness and the residue was dissolved in 25 ml. of ethanol. Suitable dilutions were made so as to give a final solution containing 10 μg. of estrone per milliliter of ethanol. Aliquots of this solution were assayed.

The results obtained with this technique are presented in Table III. The vehicle used for suspending purposes in both the commercial and control preparations was sodium chloride solution containing 5.25% gum acacia.

3. Estradiol Tablets.—Control samples were prepared consisting of crystalline estradiol in an intimate mixture with a vehicle composed of equal parts of corn starch and lactose. This mixture would appear to approximate the tablets on the market. No attempt was made to mold these control mixtures into tablet form, the extraction being made from the powder. The mixture was shaken vigorously with 25 ml. of anesthetic ether in a stoppered flask. The extract was filtered through a microsintered glass filter and evaporated to dryness. The residue was dissolved in sufficient ethanol to give a final solution containing 10 μg. of estradiol per milliliter of ethanol. Aliquots of this solution were then assayed.

An examination of a number of commercial tablets extracted by the above procedure was successful. The only change necessary in the above technique was the breaking up of the tablet into a fine powder before extraction. This was accomplished by means of a fine glass stirring rod. The results of these assays together with the results of assays of control preparations are compiled in Table IV.

TABLE III.—ASSAY OF AQUEOUS SUSPENSIONS OF ESTRONE

Preparation	Known or Stated Concn., Mg.	Determined Concn., Mg.	Recovery, %
Control	2	1.972	98.6
Control	2	2.036	101.8
Control	2	2.010	100.5
Control	2	2.000	100.0
Company A	2	1.996	99.8
Company A	2	2.058	102.9
Company A	2	2.000	100.0
Company A	2	1.992	99.6
Company A	2	1.932	96.6

TABLE IV.—ASSAY OF ESTRADIOL TABLETS

Preparation	Known or Stated Concn., Mg.	Determined Concn., Mg.	Recovery, %
Control	0.5	0.500	100.0
Control	0.5	0.500	100.0
Control	0.5	0.492	98.4
Control	0.2	0.200	100.0
Control	0.2	0.202	101.0
Control	0.2	0.199	99.5
Company C	0.5	0.497	99.4
Company C	0.5	0.506	101.2
Company C	0.5	0.502	100.4
Company C	0.2	0.200	100.0
Company C	0.2	0.201	100.5
Company C	0.2	0.199	99.5
Company D	0.1	0.101	101.0
Company D	0.1	0.100	100.0
Company D	0.1	0.100	100.0

SUMMARY

1. A satisfactory photofluorometric method for the estimation of certain crystalline estrogens has been outlined.

2. This method has been successfully adapted to the assay of commercial preparations of estrone in oil, aqueous suspensions of crystalline estrone, and estradiol tablets.

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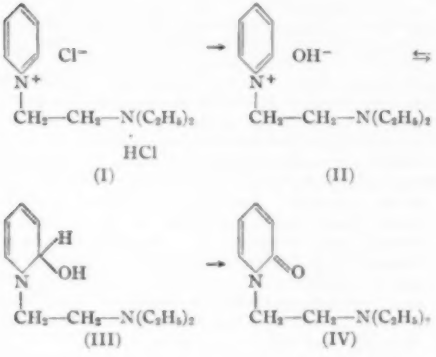
Synthesis of Some N-Dialkylaminoalkyl-4-Pyridones*

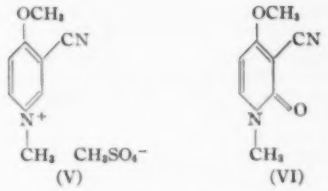
By JOHN H. SHINKAI† and LLOYD M. PARKS

Attempts to prepare some N-dialkylaminoalkyl-4-alkoxy- and 4-aryloxy-2-pyridones by oxidation of N-dialkylaminoalkyl-4-alkoxy- and 4-aryloxy-pyridinium halides with potassium ferricyanide in alkaline solution resulted instead in the formation of N-dialkylaminoalkyl-4-pyridones as a result of cleavage of the 4-alkoxy or 4-aryloxy group under the alkaline conditions used. The N-dialkylaminoalkyl-4-pyridones formed the dihydrochloride salt with HCl and the monosubstituted quaternary ammonium compounds with reactive alkyl halides.

In the synthetic investigation of compounds related in structure to the alkaloid ricinine, the preparation of certain N-dialkylaminoalkyl-4-alkoxy and 4-aryloxy-2-pyridones was studied prior to attempting the preparation of the corresponding 3-cyano derivatives which are more closely related to ricinine. Miescher and Urech (1) reported the synthesis of N-(β-diethylaminoethyl)-2-pyridone (IV) by reacting β-diethylaminoethyl chloride hydrochloride with pyridine to form N-(β-diethylaminoethyl)-pyridinium chloride hydrochloride (I) and converting this quaternary salt with aqueous alkali to the corresponding pyridinium hydroxide (II), which was oxidized with potassium ferricyanide to the 2-pyridone *via* the pseudo base (III):



In his synthesis of ricinine Reitmann (2) also reported the oxidation of N-methyl-3-cyano-4-methoxy-pyridinium methosulfate (V) to N-methyl-3-cyano-4-methoxy-2-pyridone (VI) (ricinine) with potassium ferricyanide in alkaline medium:



It was anticipated that, by oxidation of N-dialkylaminoalkyl-4-alkoxy and 4-aryloxy-pyridinium halides by the procedures described by Miescher and Urech and by Reitmann, the desired products would be obtained (Reitmann did not report the yield he obtained). However, the corresponding N-dialkylaminoalkyl-4-pyridones (VII) were obtained instead of the desired N-dialkylaminoalkyl-4-alkoxy and 4-aryloxy-2-pyridones. The conclusion that (VII) was obtained was verified by synthesis of N-(β-diethylaminoethyl)-4-pyridone from 4-hydroxypyridine by reaction with β-diethylaminoethyl chloride in aqueous alkaline medium and converting the resulting product into its dihydrochloride, which melted in the same range as the hydrochloride of (VIII) and gave no depression in a mixed melting-point test.

Several runs were made to determine at what stage the 4-phenoxy group was cleaved in the general procedure used for the attempted synthesis of N-(β-diethylaminoethyl)-4-phenoxy-2-pyridone. The results indicated that the 4-phenoxy group was hydrolyzed immediately upon addition of the sodium hydroxide solution. The same hydrolysis was effected when sodium carbonate solution was used; less than equivalent quantities of sodium carbonate gave lower yields of the hydrolysis product. Another run showed that cleavage did not occur prior to addition of the alkali.

On the basis of these results, hydrolysis of the 4-alkoxy and 4-aryloxy groups may be pictured as taking place in the following manner, with the subsequent formation of the dihydrochloride salt with HCl gas:

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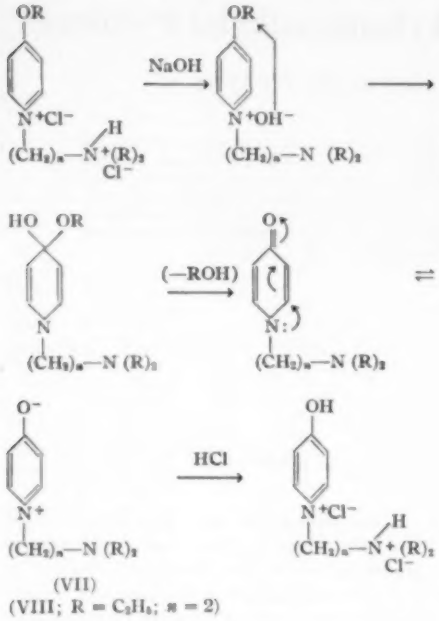
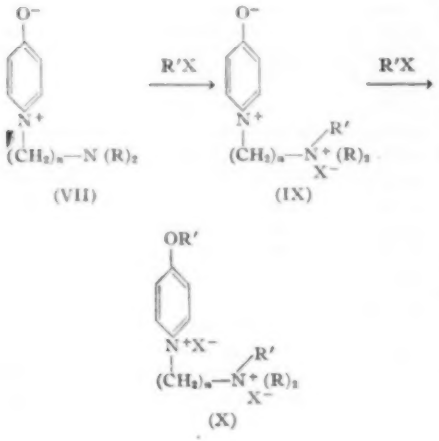
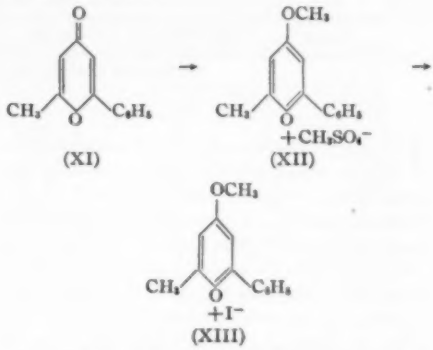


(VII)
(VIII; R = C_2H_5; n = 2)

It was of interest to determine whether these N-substituted 4-pyridones would undergo reaction with reactive alkyl halides, as well as with dimethyl sulfate, in a manner analogous to that with HCl to form the quaternary salt of the corresponding 4-alkoxy compound (X). Upon treatment of (VII) with the alkyl halide in dry benzene some quaternary compounds were obtained, but equivalent weight determinations by amperometric titration indicated that only the quaternary ammonium halide (IX) formed:



Gibson and Simonsen (3) reported that 2-phenyl-6-methyl-4-pyrone (XI) reacted with dimethyl sulfate to form the methyl methosulfate derivative (XII) which, when dissolved in water and treated with excess dilute potassium iodide solution, formed the corresponding methyl iodide derivative (XIII):



Attempts to form the methyl methosulfate derivative of (VII) gave only a gummy mass which, when treated by the method of Gibson and Simonsen for conversion to the methyl iodide derivative, yielded no solid product.

EXPERIMENTAL

4-Aryloxypyridines (4-phenoxy-, 4-*o*-tolyloxy, 4-*m*-tolyloxy, and 4-*p*-tolyloxypyridine) were made by the method described by Koenigs and Greiner (4), and by Renshaw and Conn (5), by reacting the sodium phenoxide with 4-pyridylpyridinium chloride in the presence of excess of the phenol. Yields averaged from 70% to 80%.

4-Alkoxypyridines (4-methoxy- and 4-ethoxypyridine) were made in yields of 52% and 27% to 43%, respectively, by reacting the sodium alkoxide with 4-chloropyridine. The latter compound was obtained from 4-hydroxypyridine by the method of Wibaut and Broekman (6). For the synthesis of 4-hydroxypyridine the hydrolysis of 4-pyridylpyridinium dichloride (4) was used. Another method used was the conversion of chelidonic acid (7) to chelidamic acid and decarboxylation of the latter to 4-hydroxypyridine (8).

Attempted Synthesis of Some N-Dialkylaminoalkyl-4-alkoxy- and 4-aryloxy-2-pyridones.—The general procedure for the attempted synthesis of these compounds was as follows: A mixture of 0.01 mole of the 4-substituted pyridine derivative (4-phenoxy-, 4-*o*-tolyloxy-, 4-*m*-tolyloxy-, 4-*p*-tolyloxy-, 4-methoxy-, or 4-ethoxypyridine) and 0.01 mole of the dialkylaminoalkyl halide hydrohalide (β-diethylaminoethyl chloride hydrochloride, β-dimethylaminoethyl bromide hydrobromide, or γ-diethylaminopropyl bromide hydrobromide) was placed in a 100-cc. round-bottomed flask equipped with a reflux condenser and heated in an oil bath at 150–155° for half an hour. The resulting viscous brownish reaction mixture, which solidified on cooling, was dis-

solved in 10 cc. of water with the aid of a little heat. The resulting brownish solution was chilled and then made alkaline by adding 10 cc. of a 20% sodium hydroxide solution (0.05 mole), whereupon the solution separated into two layers. This mixture was thoroughly chilled in an ice bath, and a solution of 6.6 Gm. potassium ferricyanide (0.02 mole) in 20 cc. of water was added dropwise while continually stirring and chilling. This reaction mixture was left standing at room temperature for three hours to insure completion of the reaction.

The resulting turbid brown reaction mixture was extracted with 100 cc. of chloroform (*n*-amyl alcohol was also used as the extraction solvent). The aqueous layer was saturated with anhydrous sodium carbonate, and this mixture was extracted with three 50-cc. portions of chloroform. The chloroform extracts were combined, the solvent distilled off, and the remaining viscous brown liquid was either converted directly to the hydrochloride salt or distilled at reduced pressure.

For the direct conversion to the hydrochloride salt, the viscous liquid from the chloroform extraction was dissolved in absolute methyl alcohol, and hydrogen chloride gas was bubbled through this solution. After allowing the methyl alcohol to evaporate, the remaining gummy residue was taken up with absolute ethyl alcohol, and the hydrochloride salt precipitated by adding acetone. The resulting white precipitate of the hydrochloride salt was filtered and dried. For further purification, this product was taken up with absolute ethyl alcohol and reprecipitated by adding acetone.

For the reduced pressure distillation of the viscous liquid from the chloroform extraction, it was transferred to a 10-cc. distilling flask equipped with a capillary ebullition tube. The distilling flask was immersed in an oil bath, the pressure lowered, and the oil bath temperature slowly raised. Results of typical runs are given in Table I.

N-(β-Diethylaminoethyl)-4-pyridone.—One and six-tenths grams of 4-hydroxypyridine nitrate salt (0.01 mole) and 1.7 Gm. of β-diethylaminoethyl chloride hydrochloride (0.01 mole) were dissolved in a solution of 1.2 Gm. sodium hydroxide (0.03 mole) in 15 cc. of water. The resulting yellow solution was gently refluxed for one hour. After cooling this reaction solution, it was saturated with anhydrous sodium carbonate and extracted with three 50-cc. portions of chloroform. These chloroform extracts were combined, the solvent distilled off, and the remaining viscous liquid transferred to a 10-cc. distilling flask for a reduced pressure distillation. About 1.2 Gm. of viscous yellow liquid distilled over at 225–228° under 2 to 3 mm. pressure.[1]

A portion of the above sample was converted to the dihydrochloride salt. The white precipitate which melted at 190 to 191.5° gave no depression in a mixed melting point determination with the dihydrochloride salt of m. p. 190.5 to 191.5° from one of the compounds obtained in the attempted synthesis of N-(β-diethylaminoethyl)-4-phenoxy-2-pyridone.

Reaction of N-Dialkylaminoalkyl-4-pyridones with Reactive Alkyl Halides.—The general procedure was as follows: To 10 cc. of dry benzene (dried over sodium wire) in a 25-cc. flask was added 0.003 mole of the N-dialkylaminoalkyl-4-pyridone and an excess (0.012 mole) of the alkyl halide (methyl iodide, allyl bromide, or benzyl chloride). This flask was stoppered and allowed to stand at room temperature for four days. The resulting white precipitate or gummy product was separated from the benzene solution by filtration or decantation. This product was purified from a mixture of ethyl alcohol and ethyl acetate. The results of the various runs are given in Table II.

When the above procedure was repeated using dimethyl sulfate, a thick, gummy, brownish liquid settled out from the benzene solution, but it could not be crystallized nor precipitated from the usual solvents or mixtures of them. In an attempt to form the corresponding methyl iodide derivative, a portion of this product was dissolved in a little water, and an excess of dilute potassium iodide solution was added. This solution was chilled for one week, but no product crystallized or separated out.

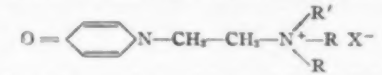
[1] Miescher and Urech (1) reported the N-(β-diethylaminoethyl)-4-pyridone to be a yellow thick oil which boils at 175° at 0.03 mm. pressure.

TABLE I.—N-DIALKYLAMINOALKYL-4-PYRIDONES

R	Yield, %	B. P., °C./Mm.	M. P. of Dihydrochloride, ° C.
$-CH_2-CH_2-N(Et)_2$[d]	62–77	224–227/2	190–191[a]
$-CH_2-CH_2-N(Me)_2$	54–78	201–204/1	264–265.5[b]
$-CH_2-CH_2-CH_2-N(Et)_2$	34–63	235–239/2	166–168[c]

[a] Calcd. for $C_{11}H_{18}ON_2Cl_2$: C, 49.43; H, 7.54; N, 10.48; equiv. wt., 133.6. Found: C, 49.48; H, 7.32; N, 10.66; equiv. wt., 136.6.
[b] Calcd. for $C_8H_{18}ON_2Cl_2$: N, 11.72; equiv. wt., 119.5. Found: N, 12.35, 12.16; equiv. wt., 121.
[c] Calcd. for $C_{13}H_{22}ON_2Cl_2$: N, 9.93; equiv. wt., 140.6. Found: N, 10.32, 10.23; equiv. wt., 139.
Equivalent weight determinations were made by amperometric titration with 0.01 *N* $AgNO_3$, using a rotating platinum and a calomel electrode.
[d] Miescher and Urech (see footnote 1).

TABLE II.—N-(β-DIALKYLAMINOETHYL)-4-PYRIDONE ALKYL HALIDES



R	R′X	Yield, %	Equiv. Wt. Calcd.	Equiv. Wt. Found	M. P., ° C.
Ethyl-	Methyl iodide	82	336.2	330	198–199.5
Ethyl-	Allyl bromide	83	315.3	...	198.5–201
Methyl-	Methyl iodide	82	308.1	...	239–241
Methyl-	Allyl bromide	82	287.2	272	179–181
Methyl-	Benzyl chloride	81	292.7	301	201.5–202.5

SUMMARY

1. Various unsuccessful attempts were made to synthesize some N-dialkylaminoalkyl-4-alkoxy- and 4-aryloxy-2-pyridones.

2. In each instance the N-dialkylaminoalkyl-4-pyridone was obtained as a result of hydrolysis of the 4-alkoxy or 4-aryloxy group under the alkaline condition employed in this investigation.

3. These N-dialkylaminoalkyl-4-pyridones reacted with hydrogen chloride gas to form the dihydrochloride salt.

4. These N-dialkylaminoalkyl-4-pyridones reacted with reactive alkyl halides to form the monosubstituted quaternary ammonium compounds.

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The Synthesis of Mercurated 8-Allyloxycaffeine and Mercurated 1,3,7-Trimethyl-9-Allyluric Acid, and a Study of Their Toxicity and Diuretic Activity*

By GAIL A. WIESE† and JAMES W. JONES‡

Two new organic mercurials, mercurated 1,3,7-trimethyl-9-allyluric acid and mercurated 8-allyloxycaffeine, were prepared and compared as to toxicity and diuretic activity. The mercurials prepared and tested are isomers, and differ in the manner in which the mercurated side chain is attached to the heterocyclic nucleus. Experiments on rats and mice showed that the mercurated uric acid derivative produced diuresis while the caffeine derivative produced no significant diuresis.

IT HAS been stated (1) that diuretic-type mercurials possess a common structure in that they consist of a cyclic nucleus with a mercurated side chain joined through an amide linkage. Numerous compounds of this type have been prepared and reported, some of which are now in clinical use. However, in reviewing the literature it was found that there were but a few instances of isomeric mercurials being compared as to toxicity and diuretic properties (2,3). It seemed of interest then to prepare two isomeric mercurials, 1,3,7-trimethyl-9-(β-methoxy-γ-hydroxymercuri)propyluric acid and 8-(β-methoxy-γ-hydroxymercuri)propoxycaffeine, the former of the diuretic type and the latter not a diuretic type, and to compare their toxicity and diuretic properties. The former compound is a diuretic-type mercu-

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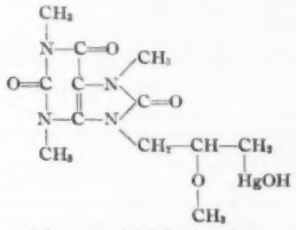
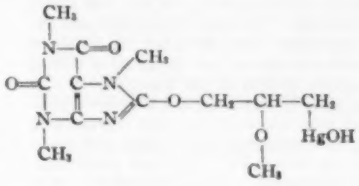
rial as it represents a mercurated side chain attached to an amide nitrogen which is part of a heterocyclic nucleus,and will henceforth be designated as mercurated 1,3,7-trimethyl-9-allyluric acid. The latter compound does not answer the description of a diuretic-type mercurial as the mercurated side chain is attached to a carbon atom through an ether linkage. This compound will hereafter be designated as mercurated 8-allyloxycaffeine. The mercurated compounds above are represented by the following formulas.



Mercurated 1,3,7-trimethyl-9-allyluric acid



Mercurated 8-allyloxycaffeine

EXPERIMENTAL

Preparation of Mercurated 8-Allyloxycaffeine.—The method of Fischer and Reese (4) was used to prepare 8-chlorocaffeine from caffeine. The method of Huston and Allen (5) was used to prepare 8-allyloxycaffeine from 8-chlorocaffeine and allyl alcohol.[1]

A solution of 25.1 Gm. (0.1 mole) of 8-allyloxycaffeine, previously dried at 110°, in 100 cc. of absolute methanol and a solution of 31.8 Gm. of mercuric acetate in 100 cc. of absolute methanol containing 0.5 cc. of glacial acetic acid were combined and refluxed on the water bath for two hours. The solution was then allowed to stand at room temperature overnight and on testing a small portion with 10% sodium hydroxide solution it gave no precipitate of mercuric oxide, indicating completeness of the reaction. The methanol was then removed by distillation under reduced pressure and a portion of the residue was recrystallized from ethanol and dried with a current of warm air. The melting point obtained for the acetoxymercuric compound was 164–165°. On standing at room temperature for two hours, the melting point was lowered to 151°, indicating decomposition in air. The remainder of the residue was suspended in 500 cc. of water, boiled for three hours, and allowed to stand at room temperature overnight. The white precipitate formed was filtered off by suction and, when dried at room temperature, it melted at 134–136°. However, when dried in the oven at 120° the melting point was 211–213°. After drying, over concentrated sulfuric acid for forty-eight hours the mercurated 8-allyloxycaffeine was analyzed for mercury.

Anal.—Calcd. for $C_{12}H_{15}HgN_4O_5$: Hg, 40.21%. Found: Hg, 40.21%; 40.24%.

[1] Furnished by the Shell Chemical Corporation.

Preparation of Mercurated 1,3,7-Trimethyl-9-allyluric Acid.—1,3,7-Trimethyl-9-allyluric acid was prepared by two methods. The first method was by a series of reactions starting with 1,3-dimethylbarbituric acid, which was prepared from dimethylurea and diethyl malonate, according to directions in "Organic Syntheses" (6). Using the method of Bobranski and Synowiedski (7), 1,3-dimethylvioluric acid was prepared and reduced with zinc and sulfuric acid to 1,3-dimethyl-9-allyluric acid by the method of Gatewood (8), with subsequent methylation with methyl sulfate. The over-all yields with this method were less than 10%, so another method of preparing 1,3,7-trimethyl-9-allyluric acid was sought.

Wislicenus and Korber (9) found that 8-methoxy- and 8-ethoxycaffeine would rearrange to 1,3,7,9-tetramethyluric acid and 1,3,7-trimethyl-9-ethyluric acid, respectively, when heated in a sealed tube. Huston and Allen (10) noted that these caffeine ethers would rearrange in better yields when heated in an open tube. These investigators also found 8-allyloxycaffeine rearranged to 1,3,7-trimethyl-9-allyluric acid when heated in an open tube, with yields of 50%. Their method, with slight modification of temperature, was used in preparing the uric acid derivative in this investigation. Yields of 75% were obtained by heating 8-allyloxycaffeine in an open tube in an oil bath at 175–180° for five hours. Above 180° the yields diminished and below 165° there was no rearrangement.

To form the mercurated uric acid derivative, 2.5 Gm. of 1,3,7-trimethyl-9-allyluric acid in 25 cc. of hot, dry methanol was added to 3.18 Gm. of mercuric acetate in 30 cc. of dry methanol, and the mixture allowed to stand at room temperature overnight. The methanol was partially removed by evaporation on the water bath. On cooling, crystals formed and, when recrystallized from absolute alcohol, melted at 180–181°. The yield was 4.5 Gm. or 83% of theory. The acetoxymercuric compound, when dissolved in water and boiled for three hours, and allowed to stand overnight, formed crystals melting at 176–177°. After drying over concentrated sulfuric acid for forty-eight hours the mercurated 1,3,7-trimethyl-9-allyluric acid was analyzed for mercury.

Anal.—Calcd. for $C_{13}H_{18}HgN_4O_6$: Hg, 40.21%. Found: Hg, 40.29%; 40.07%.

Toxicity Studies.—The LD_{50} of the two mercurials was determined on both mice and albino rats. The mice used weighed from 29 to 35 Gm. and the

albino rats, of mixed sex, weighed from 130 to 210 Gm. The solutions were prepared so that 1 cc. contained 4 mg. of compound, or 1.6 mg. of mercury. Mercurated 1,3,7-trimethyl-9-allyluric acid was easily soluble in this strength, but mercurated 8-allyloxycaffeine had to be mixed with an equal weight of sodium salicylate to obtain a clear solution.

The solutions were injected intraperitoneally and the deaths within twenty-four hours recorded. The method of probits, as described by Mather (11), was used to estimate the LD_{50} of the test compounds. A total of 60 rats and 72 mice was used in the toxicity studies. The LD_{50} of mercurated 8-allyloxycaffeine was found to be that representing about 20 mg. of mercury per Kg. in rats, and about 39 mg./Kg. in mice. The dose of mercury and the percentage mortality are tabulated in Tables I and II. The dose of mercury per kilogram of animal is plotted against probits in the manner shown in Fig. 1. The LD_{50} of mercurated 1,3,7-trimethyl-9-allyluric acid was found to be that representing about 28 mg. of mercury per Kg. in mice, and about 18 mg./Kg. in rats. The dose and percentage mortality are tabulated in Tables III and IV. The dose of mercury per kilogram of animal is plotted against probits in the manner shown in Fig. 1.

Diuretic Activity Studies.—A modification of the method applied by Lipschitz (12), using albino rats, was employed for determining the optimum diuretic dose and the diuretic activity of the test compounds.

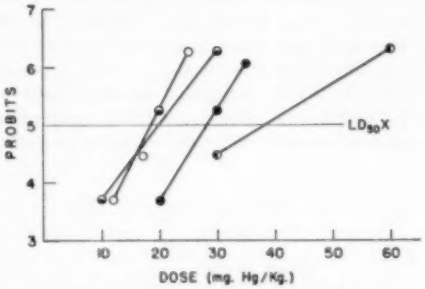


Fig. 1.—LD_{50} determination of mercurated 1,3,7-trimethyl-9-allyluric acid and mercurated 8-allyloxycaffeine on rats and mice.
○ Mercurated 1,3,7-trimethyl-9-allyluric acid on rats. ● Mercurated 1,3,7-trimethyl-9-allyluric acid on mice. ◒ Mercurated 8-allyloxycaffeine on rats. ◐ Mercurated 8-allyloxycaffeine on mice.

TABLE I.—LD_{50} DETERMINATION OF MERCURATED 8-ALLYLOXYCAFFEINE ON RATS[a] (INTRAPERITONEAL)

Dose in Mg. Hg/Kg.	No. Rats Used	Deaths	Mortality, %	Curve, %	Deviate	Probit
30	10	9	90	40	1.28 + 5	6.28
20	10	6	60	10	0.25 + 5	5.25
10	10	1	10	40	−1.28 + 5	3.72

[a] Estimated LD_{50} 20 mg. Hg/Kg. (see Fig. 1).

TABLE II.—LD_{50} DETERMINATION OF MERCURATED 8-ALLYLOXYCAFFEINE ON MICE[a] (INTRAPERITONEAL)

Dose in Mg. Hg/Kg.	No. Mice Used	Deaths	Mortality, %	Curve, %	Deviate	Probit
60	10	9	90	40	1.28 + 5	6.28
50	10	5	50	0	0.00 + 5	5.00
30	10	3	30	20	−0.52 + 5	4.48

[a] Estimated LD_{50} 39 mg. Hg/Kg. (see Fig. 1).

TABLE III.—LD_{50} DETERMINATION OF MERCURATED 1,3,7-TRIMETHYL-9-ALLYLURIC ACID ON RATS[a] (INTRAPERITONEAL)

Dose in Mg. Hg/Kg.	No. Rats Used	Deaths	Mortality, %	Curve, %	Deviate	Probit
25	10	9	90	40	1.28 + 5	6.28
17	10	3	30	20	−0.52 + 5	4.48
12	10	1	10	40	−1.28 + 5	3.72

[a] Estimated LD_{50} 18 mg. Hg/Kg. (see Fig. 1).

TABLE IV.—LD_{50} DETERMINATION OF MERCURATED 1,3,7-TRIMETHYL-9-ALLYLURIC ACID ON MICE[a] (INTRAPERITONEAL)

Dose in Mg. Hg/Kg.	No. Mice Used	Deaths	Mortality, %	Curve, %	Deviate	Probit
35	14	12	85.7	35.7	1.07 + 5	6.07
30	15	9	60	10	0.25 + 5	5.25
20	10	1	10	40	−1.29 + 5	3.71

[a] Estimated $L\,D_{50}$ 28 mg. Hg/Kg. (see Fig. 1).

Food and water were withheld from the animals for twelve hours. Sixty animals were divided into twelve groups of five animals each. Each animal was fed 25 cc. of normal saline per Kg. by stomach tube. One group, which served as a control, received no drug and another group received Mersalyl with Theophylline in doses corresponding to 8 mg. of mercury per Kg., intraperitoneally. Five groups of animals received doses of mercurated 8-allyloxycaffeine corresponding to 2, 4, 6, 8, and 10 mg. of mercury per Kg. with an equal weight of sodium salicylate. Five groups were given corresponding doses of mercurated 1,3,7-trimethyl-9-allyluric acid. The urine excreted by each group of animals within a twenty-four hour period was collected, the volume excreted being recorded each hour during the first five hours, after which ten, twelve and twenty-four hour recordings were made. The total volume of urine was transposed to urinary excretion by the following ratio:

$$\text{Urinary excretion} = \frac{\text{Volume urine collected} \times 100}{\text{Volume liquid administered}}$$

The hourly volumes recorded were transposed to per cent hourly excretion for each dose of each compound tested and for saline and Mersalyl with Theophylline by the following ratio:

$$\text{Per cent hourly excretion} = \frac{\text{Hourly urine volume} \times 100}{\text{Total volume urine collected}}$$

The diuretic activity of each compound at each dose level was computed by the following formula:

$$\text{Diuretic activity} = \frac{\text{Urinary excretion of test}}{\text{Urinary excretion of control}}$$

The hourly excretion data for the various doses of the mercurials are tabulated in Tables V and VI.

The optimum diuretic dose of mercurated 1,3,7-trimethyl-9-allyluric acid was found to be that corresponding to 6 mg. of mercury per Kg. At this dose the compound had a diuretic activity of 1.56. In the case of mercurated 8-allyloxycaffeine, the optimum diuretic dose was found to be that corresponding to 2 mg. of mercury per Kg. However, the diuretic activity was insignificant. This data is tabulated in Tables VII and VIII.

DISCUSSION

These experiments have shown that the two isomeric organic mercurials differ considerably in their effect on urine excretion. The new diuretic-type mercurial, mercurated 1,3,7-trimethyl-9-allyluric acid produces diuresis in doses corresponding to

TABLE V.—PER CENT HOURLY URINE EXCRETION FROM SINGLE INTRAPERITONEAL DOSES OF MERCURATED 1,3,7-TRIMETHYL-9-ALLYLURIC ACID

Drug	Dose in Mg. Hg/Kg.	Total Urine Excreted by Hours, %						
		1	2	3	4	5	12	24
Saline	Control	15.2	19.2	23	27	30	65	100
Mersalyl with Theophylline	8	17.2	22.4	34.4	36.2	41.3	65.5	100
Mercurated 1,3,7-Trimethyl-9-Allyluric Acid	2	11	14	17	20	20	55	100
Mercurated 1,3,7-Trimethyl-9-Allyluric Acid	4	15.5	26.6	35.5	48.8	51	71.1	100
Mercurated 1,3,7-Trimethyl-9-Allyluric Acid	6[a]	21	25	34.6	44	55	77	100
Mercurated 1,3,7-Trimethyl-9-Allyluric Acid	8	15.9	22.7	31.8	45.5	59	86.4	100
Mercurated 1,3,7-Trimethyl-9-Allyluric Acid	10	10	12	12	17	20	46	100

[a] Optimum diuretic dose.

TABLE VI.—PER CENT HOURLY URINE EXCRETION FROM SINGLE INTRAPERITONEAL DOSES OF MERCURATED 8-ALLYLOXYCAFFEINE

Drug	Dose in Mg. Hg/Kg.	Total Urine Excreted by Hours, %						
		1	2	3	4	5	12	24
Saline	Control	10	24	31	33	38	69	100
Mersalyl with Theophylline	8	17.2	22.4	34.4	36.2	41.3	65.5	100
Mercurated 8-Allyloxycaffeine	2[a]	8	8	25	42	54	67	100
Mercurated 8-Allyloxycaffeine	4	6	7.5	9	15	27	60	100
Mercurated 8-Allyloxycaffeine	6	8.6	8.6	13	14	20	57	100
Mercurated 8-Allyloxycaffeine	8	4	8	8	8	12.5	42	100
Mercurated 8-Allyloxycaffeine	10	0	0	0	0	0	30	100

[a] Optimum diuretic dose.

TABLE VII.—DIURETIC ACTIVITY OF VARIED DOSES OF MERCURATED 8-ALLYLOXYCAFFEINE ON RATS

Dose in Mg. Hg/Kg.	No. Animals Used	Volume Saline Administered, Cc.	Volume Urine Collected, Cc.	Excretion, %	Excretion Control, %	Diuretic Activity
2	4	15.5	12	77.4	75	1.03
4	4	19.5	16.5	84.6	116	0.73
6	4	18.25	17.5	96	116	0.83
8	4	21	12	57	116	0.49
10	4	18.5	6.5	36	75	0.48

TABLE VIII.—DIURETIC ACTIVITY OF VARIED DOSES OF MERCURATED 1,3,7-TRIMETHYL-9-ALLYLURIC ACID ON RATS

Dose in Mg. Hg/Kg.	No. Animals Used	Volume Saline Administered, Cc.	Volume Urine Collected, Cc.	Excretion, %	Excretion Control, %	Diuretic Activity
2	4	20.5	18	90	75	1.2
4	4	19.5	22.5	115.3	82.5	1.39
6	4	20.25	26	128.4	82.5	1.56
8	4	18.5	22	119	82.5	1.44
10	4	19.75	12	60	75	0.8

2, 4, 6, and 8 mg. of Hg/Kg. There was a diminished diuretic effect with a dose equivalent to 8 mg. of Hg/Kg. and an inhibition of urine excretion with a dose equivalent to 10 mg. of Hg/Kg. This is in agreement with Roby and Pfeiffer (13) and Lipschitz, Hadidian, and Kerpcsar (12), who reported that mercurial diuretics cause a decrease in urine excretion in doses above the optimum diuretic dose. Mercurated 8-allyloxycaffeine, which is isomeric with the above compound, having the mercurated side chain attached to the heterocyclic nucleus through an ether oxygen, showed no increase in urinary excretion in doses representing 2 mg. of Hg/Kg. In doses representing 4, 6, 8, and 10 mg. of Hg/Kg., it showed great inhibition of urinary excretion. The highest dose, 10 mg. of Hg/Kg., inhibited urine excretion to the extent that only 36% of the liquid administered was recovered. It was of interest to note the amount of urine excreted after administering doses above 2 mg. of Hg/Kg. (Table VI). The 10 mg. of Hg/Kg. dose suppressed all urinary excretion for the first five hours. With doses of 4, 6, and 8 mg. of Hg/Kg., the per cent excretion was less for the first five hours than for the control group. However, the 2 mg. of Hg/Kg. dose caused more than 50% of the total urine to be excreted during the first five hours. Mercurated 1,3,7-trimethyl-9-allyluric acid exhibited this same property with doses of 4, 6, and 8 mg. of Hg/Kg. (Table V). Hunt, Walter, and Fosbinder (1), in studying absorption and excretion of mercury compounds, found that the relative position of the mercury did not significantly influence the rate of absorption of the compound from the site of injection, but did influence the retention of mercury. In these experiments there was some evidence of mercurial retention in animals receiving mercurated 8-allyloxycaffeine, shown by slow recovery of the animals surviving high doses. This toxicity was not observed with high doses of mercurated 1,3,7-trimethyl-9-allyluric acid and the animals surviving high doses showed quick recovery.

The foregoing pharmacological studies are not to be interpreted as complete, but rather as screening tests to determine whether or not the compounds had possible usefulness as diuretics.

SUMMARY

1. Two new isomeric mercurials, mercurated 8-allyloxycaffeine and mercurated 1,3,7-trimethyl-9-allyluric acid were prepared and a comparison of their toxicity and diuretic properties was made.

2. The nondiuretic-type mercurial, mercurated 8-allyloxycaffeine appeared to have a somewhat lower acute toxicity than the diuretic-type mercurial, mercurated 1,3,7-trimethyl-9-allyluric acid.

3. Mercurated 8-allyloxycaffeine inhibited urine excretion in all but the lowest dose given (2 mg. Hg/Kg.). Mercurated 1,3,7-trimethyl-9-allyluric acid caused diuresis in doses as large as 8 mg. of Hg/Kg. Antidiuresis was observed in higher doses.

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The Bactericidal Action of Triethylene Glycol on Air Filters of Various Types*

By SAMUEL H. HOPPER†

It has been demonstrated that hair and glass filters impregnated with triethylene glycol reduced the number of bacteria put on the filters by over 99 per cent under laboratory conditions. Treatment of furnace filters with triethylene glycol produced reduction of 75 per cent in bacterial content of the air as measured by settling plates. The use of glycolized filters has not been demonstrated to effect the incidence of respiratory disease under the conditions of use, and the system is not recommended for general use in public meeting places until further work has been done.

A LARGE group of investigators has reported on the use of triethylene glycol vapor as an aid in air sanitation (1). For best results, the amount of glycol vapor must be carefully controlled in relation to the relative humidity at any given temperature (2). A method is reported here which may be called the reverse of the process described above. The glycol is put on a furnace-type filter and the air of an enclosed space is recirculated through the glycolized filter. This method apparently collects the air-borne organisms and a bactericidal action takes place on the fibers or in the interstices of the filter.

PROCEDURE

The same type of filter as used commonly in furnaces or in air-conditioning equipment was adapted for this study. One ounce of triethylene glycol was painted onto the 10-inch square filter, which was then fitted into a rectangular tunnel as shown in Fig. 1. The arrangement was such that air was blown through by means of a fan having a capacity of 500 cubic feet per minute. Filters made of hog hair[1] and of fiberglas[2] were used. The test organisms were *Staphylococcus aureus* and *Serratia marcescens*. These were grown in pure culture at 37° on Difco nutrient broth and transplanted daily. A twenty-four-hour culture of the test organisms was atomized onto the outlet side of the filter with a DeVilbiss No. 15 atomizer and a No. 6 bulb. Immediately after atomization a 1-Gm. sample of the filter was removed aseptically, and plated out in Difco nutrient agar using the serial dilution technique. Counts were made twenty-four hours after incubation at 37° with all experiments in duplicate.

After the sample had been taken the filter was inserted in the boxlike arrangement and the fan was started. Samples were taken at regular intervals and the air coming out of the filter was tested with an electrostatic bacterial air sampler[3] as shown in Fig. 2.

EXPERIMENTAL AND DISCUSSION

1. Using a hog hair filter impregnated with one ounce of a purified petroleum oil supplied by the manufacturer, organisms were sprayed on and the following results obtained. With *Staphylococcus aureus* in duplicate experiments, the average count at the start was 75×10^6 and 160×10^6, respectively. After three hours with the fan going constantly, the counts were 45×10^6 and 104×10^6, respectively, or a reduction of 40% in total plate count in the first case and 35% in the second.

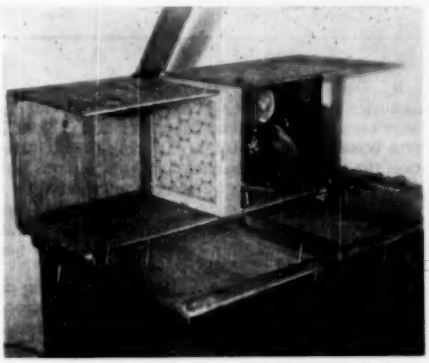

Figure 1

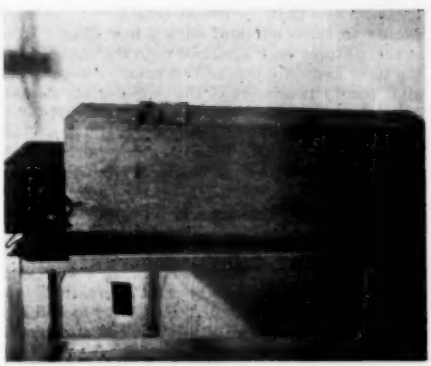

Figure 2

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[1] Kindly supplied by the Wilson Packing Co., Chicago, Ill.
[2] Kindly supplied by the Owens-Corning Fiberglas Corp., Newark, Ohio.
[3] Luckiesh-Holladay-Taylor Electrostatic Bacterial Air Sampler. Lighting Research Laboratory, General Electric Co., Nela Park, Cleveland, Ohio.

With *Serratia marcescens* in similar experiments the counts at the start were 87×10^6 and 280×10^6, respectively. After three hours the counts were 40×10^6 and 153×10^6, respectively, or reductions of approximately 95% in each case.

2. The experiments described above were repeated with filters impregnated with triethylene glycol, with results as noted in Table I. With either organism the glycol-treated filter gave reductions in bacterial count of 99% plus.

If the glycol is painted onto the inlet side of the filter and organisms are subsequently sprayed onto that same side, the result is a reduction of 99% plus. There is no difference in per cent reduction in bacterial count based upon whether the filter is painted with glycol on the inlet or outlet side. It was thought that using the outlet side of the filter gave this system its most severe test, and that method was used in all subsequent work.

TABLE I.—PER CENT REDUCTION BY PLATE COUNT—HAIR FILTER

	Staphylococcus aureus	*Serratia marcescens*
Outlet side		
Oil	37	95
Glycol	99+	99+
Inlet side		
Glycol	99+	99+

3. Using glycolized filters the organisms were atomized onto the inlet side of the filter and samples were taken in the usual manner. Reduction of 99% plus occurred here as before. During the atomization the electrostatic bacterial air sampler was put directly in the outgoing air stream and showed that innumerable bacteria came through at that time. Within one to two hours, however, the plates on the air sampler showed only one or two colonies per plate.

4. The glass filters normally have a coating of tricresylphosphate. Using the method as outlined, filters of plain glass, glass with triethylene glycol, glass with tricresylphosphate, and glass with triethylene glycol and tricresylphosphate were used. The results are shown in Table II. Note that tricresylphosphate worked as well as glycol with either organism and that the results with glycol alone are similar to those obtained with a hair filter. Here again, cultures were atomized onto the inlet side of the filter and only one to two colonies were found after one to two hours in the outgoing air stream, using the bacterial air sampler (see Table III).

5. The length of time the glycol is active is of interest. In one case a hair filter was painted with glycol and allowed to stand at room temperature for two weeks. It was then put into the test machine and the motor ran continuously for thirty-two hours. At that time, using *S. aureus* as the test organism, the reduction in count was 99% plus. The filter was put back into the machine and the fan was started again for an additional forty-eight hours, making a total of eighty hours in all. The glycol looked dry and the filter was very dirty. Using *S. aureus*, the per cent reduction was still over 99. A glass filter with glycol, after two hundred and sixteen hours of use, gave reductions of over 99% with *S. aureus*.

6. A glass filter treated with glycol was put into the experimental machine and the air of a room containing 2700 cubic feet was continuously recirculated. At 500 cubic feet per minute it took about five and one-half minutes to pass all of the air of the room through the filter. The fan was run for one hour and organisms were then sprayed into the air.

TABLE II.—PER CENT REDUCTION BY PLATE COUNT—GLASS FILTER

	Staphylococcus aureus	*Serratia marcescens*
Tricresyl phosphate	97	99
Tricresyl phosphate with glycol	99+	99+
Glycol	99+	99+
Plain glass	77	87

The rate at which they died was the same as that for controls. This indicates that if the filter-fan arrangement puts glycol vapor in the air, it does not put out enough vapor to effect a reduction in bacterial count. Work with the bacterial air sampler had previously shown that organisms are not blown out into the air, and this experiment aids in concluding that the reduction of the number of organisms takes place either on the filter or in the interstices of the filter.

TABLE III.—ATOMIZATION OF CULTURE TO INLET SIDE—HAIR OR GLASS FILTER WITH TRIETHYLENE GLYCOL

Time	Plate Count
During atomization	Too many to count
1 Hour after atomization	1 to 2 colonies/plate
2 Hours after atomization	1 to 2 colonies/plate

7. Next a glycolized filter was fitted under a fan in a manner as shown by Fig. 3. When glycol was applied to the filter the test organism, *S. marcescens*, practically disappeared from the air of the room (see Fig. 4).

8. At this time the technique of using a glycolized filter was taken out of the laboratory and applied in

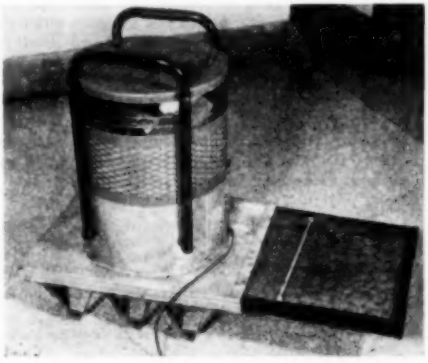

Figure 3

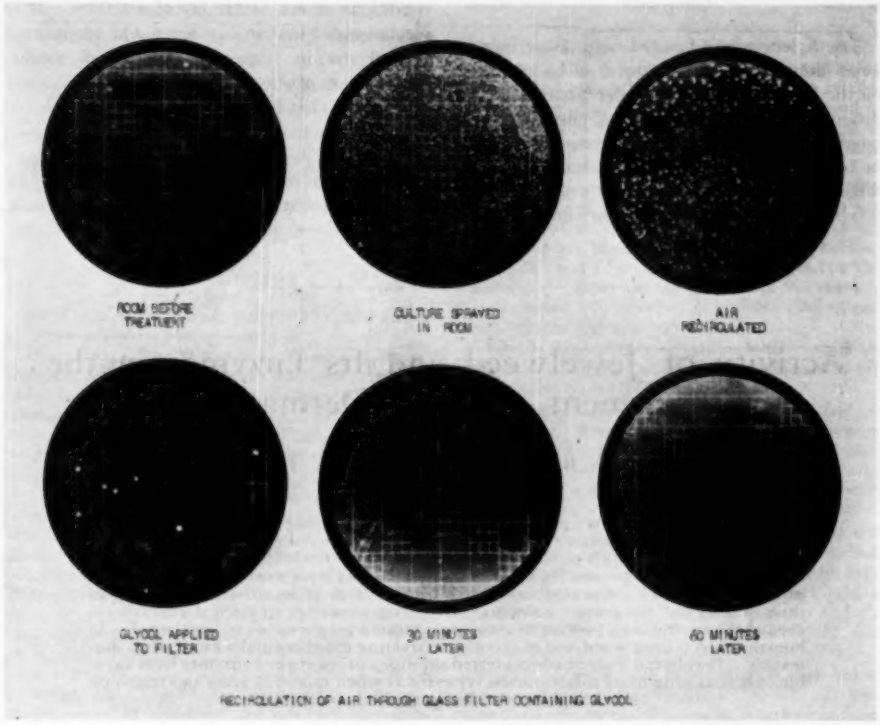

Figure 4

each of two business establishments. Number 1 was an advertising agency located in a house which had been redecorated and made into a business arrangement. Table IV shows how the application of glycol to the filters of a furnace with a blower attachment brought the count down by about 75%, using fifteen-minute settling plates. There was no attempt to control the relative humidity and the furnace did not have a humidifier. This was done during the fall and early winter.

Counts represent the total of six rooms. There were no restrictions on the opening and closing of doors and windows, or any change in janitorial service, i.e., ordinary working conditions prevailed.

Table V summarizes the work in a veterinarian's place of business. The reduction in count here was approximately 75% despite constant opening and closing of doors to bring in dogs for treatment.

TABLE IV.—BUSINESS ESTABLISHMENT NO. 1

Time	Plate Count
Start	219
2 days	300
Treated with glycol	
6 days	65
51 days	31
81 days	56

TABLE V.—BUSINESS ESTABLISHMENT NO. 2

Time	Plate Count
Start (no glycol)	534
3 days later (glycol)	461
8 days later	351
30 days later	129
Inserted new filters treated with glycol	
60 days later	148

The general reduction in count of 75% is desirable, but whether it is enough of a reduction to have any effect on the incidence of respiratory disease is a moot question. The use of settling plates rather than an aspirator type system for measurement of the air-borne organisms effects the per cent reduction in count (3). It is not known how much disease, and in particular diseases of the respiratory tract, is caused by air-borne organisms. Therefore, it would neither be logical nor correct to propose that a reduction of 75% in the number of air-borne organisms in these business establishments had any effect upon the health of the people exposed to these conditions. Until proof is available it is felt that a reduction in the number of air-borne germs is desirable, but no recommendation is made for the adoption of this method generally in homes, offices, school rooms, theaters, or other places where groups of people convene.

SUMMARY

Triethylene glycol painted onto either hair or glass filters reduced the number of bacteria put on the filters by over 99% under laboratory conditions. In each of two places of business, treatment of the furnace filters gave reduction of 75% in bacterial content of the air as measured by settling plates. No evidence is presented concerning the incidence of respiratory disease under conditions of use of the glycolized filter. It is recommended that this system not be adopted for general use in homes, offices, school rooms, theaters, or other public meeting places until further work has been done.

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Activity of Jewelweed and Its Enzymes in the Treatment of Rhus Dermatitis*,†

By MELVIN R. GIBSON‡ and FRANK T. MAHER§

In sensitized albino guinea pigs it was not possible to demonstrate therapeutic activity for jewelweed in poison ivy dermatitis after the outward manifestations became apparent. Neither was it possible to exhibit activity in limiting the biologic activity of poison ivy when poison ivy extracts and jewelweed juice were combined *in vitro*, or on the skin. This was attributable to a lesser degree of sensitivity in guinea pigs than in man and the greater amounts of ivy toxin necessary to elicit dermatitis exceeded the neutralizing powers of even concentrated preparations of jewelweed. In human subjects jewelweed was of no value in treating experimentally established dermatitis. Jewelweed juice or concentrated solutions of jewelweed enzymes were capable of inactivating more dilute poison ivy extracts when mixed *in vitro* and tested on human subjects under controlled conditions.

REFERENCES to the therapeutic activity of the several species of *Impatiens*, more commonly known as "jewelweed," have been found in medical literature for nearly one hundred years. It has been employed most frequently in the treatment of contact dermatitis caused by the plant *Rhus radicans* L. (1–10). Adequately controlled studies have not been heretofore reported, and this therapy has therefore remained empirical and subject to dispute.

Gisvold (11) identified a peroxidase and a polyphenol oxidase in *Impatiens capensis* Meerb. He noted that the quantity of phenols in hydroalcoholic solutions of the active principle of poison ivy was markedly decreased by admixture for several hours with jewelweed juice. He indicated that the oxidases might be affecting the poisonous phenols in poison ivy extract (12, 13) and suggested that jewelweed activity in the treatment of poison ivy dermatitis was due to the oxidases present. Recent studies (14, 15) have indicated that phenol-oxidizing enzymes may inactivate the active principle of *Rhus radicans* L. Such enzymes are widely distributed in the plant kingdom (16–19). The widespread use of plant juices among the laity for the treatment of poison ivy dermatitis is noted by McNair (20) who lists one hundred and nine remedies of plant nature. Sizer and Prokesch (15) suggested that these plant juice remedies might be applications of the phenolase activity similarly widespread in plants.

This investigation had for its primary purpose a study of the phenol-oxidizing enzymes of species of *Impatiens*. This study was to correlate the activity of these enzymes with the attributed activity of extracts of *Impatiens* species against induced Rhus dermatitis in guinea pigs and in human subjects.

Over three hundred and fifty pounds of *Impatiens pallida* Nutt. and *Impatiens capensis* Meerb. were gathered in September of 1947 and 1948. Species were handled separately. Using the spectrophotometric method of quantitating

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polyphenolase and peroxidase activity, the basic physical and chemical properties of the enzymes of jewelweed were determined and the partial isolation of the enzymes accomplished. These methods of assay and the techniques used in the partial isolation of the enzymes comprise another paper by the authors. The purified enzyme preparations made possible a study of the activity of these and the jewelweed juice itself in the treatment of experimental poison ivy dermatitis.

EXPERIMENTAL

Studies were made using sixteen sensitized albino guinea pigs and nine human subjects.

The poison ivy extract used to induce the dermatitis was prepared from leaves and stems of *Rhus radicans* L. in a manner similar to that of Simon and Lotspeich (21), using fresh plant material which had been kept frozen in sealed plastic bags. The extract so obtained was diluted or concentrated to obtain weight to volume dilutions of 1:10, 1:25, 1:50, 1:100, 1:500, 1:1000, 1:10,000, and 1:50,000.

Animal Experiments.—During the experiments the patch test areas were covered with thick oval bunion pads and wrapped with elastic bandage over a cotton foundation using a technique of the authors (22). The guinea pigs were prepared for the skin tests by clipping followed by shaving or depilation with the depilatory of Pitesky and Last (23). Areas of exposure were approximately 1 cm. in diameter. One side of the animal served as a control, the other as a test site. The bunion pad reservoir cap technique provided chambers for the wet dressings. The animals were sensitized to poison ivy following the method of Simon and co-workers (24), using the 1:10 concentration of poison ivy extract.

Fifty-two cases of Rhus dermatitis in guinea pigs which had been exposed for twenty-four hours were treated with jewelweed juice applied regularly during the progress of the dermatitis. No observable differences could be discerned between test and control areas.

Twelve controlled experiments were conducted to determine the *in vitro* inactivation of the poison ivy with jewelweed. The 1:10 poison ivy extract was mixed with an equal quantity of jewelweed juice for the test site and with an equal quantity of distilled water for the control site. These mixtures were allowed to stand for twenty-four hours before application and removed after twenty-four hours of exposure. An equal degree of dermatitis was observed in both areas.

Lyophilized juice was used in an attempt to build up the jewelweed concentration in proportion to the poison ivy extract. A mixture eight times as concentrated as the jewelweed juice itself was used and will be referred to as concentrated jewelweed juice. When this material was fully reconstituted it was found to have enzyme activity approximating raw juice indicating little loss in activity by the lyophilization process.

Eleven controlled experiments were conducted, exposing the guinea pigs to the 1:25 poison ivy extract. Immediately after applying poison ivy, a concentrated jewelweed juice solution was applied to the test site, and distilled water was applied to the control site. After twenty-four hours, activity of jewelweed was observed in diminishing the severity of the dermatitis produced.

Eleven controlled experiments were conducted testing for *in vitro* inactivation of the poison ivy extract using concentrated jewelweed juice buffered to the optimum *p*H for enzymic activity. Two cc. of 1:10 poison ivy extract was mixed with an equal quantity of concentrated jewelweed solution to which was added 1 cc. of *p*H 7.3 phosphate buffer and ten drops of 3% hydrogen peroxide. This was allowed to stand overnight. The solution was applied to the test site and a control solution substituting distilled water for the jewelweed was used for the control site. After twenty-four hours the extracts were removed. No differences between control and test sites were detectable.

Human Experiments.—Nine volunteer students were submitted to repeated exposure to poison ivy extracts over a period of four months.

The ulnar surface of the forearm was used as the test site. Areas of exposure to poison ivy were covered with the reservoir bunion pads held in place with small amounts of adhesive tape. The caps were covered with a layer of cotton and bandaged with gauze and tape.

For application every twenty-four hours, the wet dressings were placed in the reservoirs as described for the guinea pigs. For wet dressings to be kept saturated by regular applications of the liquid, a small rubber tube was fitted into the cap and extended through the bandage. The liquid was dropped into the test site through this rubber tube.

Human subjects have been found to vary widely in their sensitivity to poison ivy (21, 25). To prevent the development of excessive reactions each individual was exposed in two areas 1 cm. in diameter to dilutions 1:50,000, 1:5000, 1:1000, 1:500, 1:100, 1:50, 1:25, and 1:10 or to that stage in the series where dermatitis was produced. Twenty-four hour exposures were used. Individuals varied considerably in threshold sensitivities. One required only a 1:1000 dilution, others required as much as a 1:10 dilution to produce a dermatitis.

Following twenty-four hour exposures to the poison ivy extract at the concentration required to induce dermatitis in each individual, the extract was removed and jewelweed juice was applied in a manner previously described to keep the area continually wet. The control was treated with distilled water. Bandages were changed daily and areas carefully washed with water utilizing cotton swabs.

Ten cases, seven mild and three severe, were so treated for four to five days. In no case was there any indication that jewelweed accelerated the rate of healing.

The activity of jewelweed and isolated enzymes on poison ivy *in vitro* was tested. Five tests were accomplished, each in sets of three test areas per individual. Test area number one was treated with a mixture of 1 cc. of the ivy extract, at twice the concentration of the threshold of the individual, mixed with 1 cc. of distilled water, a few drops of *p*H 7.3 phosphate buffer and a few drops of 3% hydrogen peroxide, and allowed to stand for twenty-four hours. The test solution for area number two was the same as number one except jewelweed juice was substituted for water. Area number three was treated with a similar mixture except that an enzyme solu-

tion which had similar oxidative capacities toward polyphenols as jewelweed juice was used instead of jewelweed juice or water. No individual was used with a sensitivity requiring greater than a 1:50 dilution of the poison ivy to produce a reaction. Area number one in all cases produced dermatitis but two and three did not. Two tests were run on the same individuals using the boiled enzyme solution following the above procedure and dermatitis resulted in both cases.

Three tests were conducted using the same individuals but substituting the enzyme solution above diluted with three parts of water in preparing the solution for area number three. Area three showed some modification of activity. Area two was again negative. This was the rule in all three sets of experiments of this nature. This indicated that the enzyme must be of a definite oxidative power and if diluted below the optimum concentration in relation to poison ivy toxin there is a correspondingly lesser inactivation of the toxin.

The activity of jewelweed and isolated enzymes *in vivo* on poison ivy was investigated. In these experiments two areas were exposed to effective concentrations of poison ivy extract. One test area was treated immediately with concentrated jewelweed juice eight times the concentration of the natural juice, and applied in the form of a wet dressing. The control area was similarly treated with distilled water. The dressings were kept wet for twenty-four hours and removed. The results from three experiments indicated partial inactivation of the poison ivy at the site of application of the concentrated jewelweed solution.

The above experiments were repeated in four cases using a solution of partially purified and isolated jewelweed enzymes. This solution represented several times the polyphenol oxidase activity of jewelweed juice. The results obtained were similar to those obtained with the concentrated jewelweed solution and again indicated partial inactivation of the toxic constituents of the poison ivy extract.

SUMMARY

The activity of jewelweed juice and of enzymes partially isolated from this source in the treatment of experimental Rhus dermatitis has been evaluated in 16 sensitized albino guinea pigs and 9 human subjects.

In sensitized, albino guinea pigs it was not possible to demonstrate therapeutic activity for jewelweed in poison ivy dermatitis after the outward manifestation became apparent. Neither was it possible to exhibit activity in limiting the biologic activity of poison ivy when poison ivy extracts and jewelweed juice were combined *in vitro*, or on the skin. This was attributable to a lesser degree of sensitivity in guinea pigs than in man. Because of this relatively low susceptibility, the amounts of ivy toxin necessary to elicit dermatitis exceeded the neutralizing powers of even concentrated preparations of jewelweed.

It was found in human subjects that after the dermatitis became apparent, the jewelweed was of no value in its treatment. Jewelweed juice or a concentrated enzyme solution of a similar oxidative power toward polyphenols was capable of inactivating more dilute poison ivy extracts when mixed *in vitro* and tested biologically under controlled conditions. It was also found that when concentrated jewelweed juice and concentrated enzyme solutions were applied simultaneously with the poison ivy extract to the skin the enzymes and juice were capable of diminishing the toxicity of the poison ivy extract.

CONCLUSIONS

The conclusions from these studies may be briefly outlined:

1. Species of *Impatiens* are of no value under the conditions investigated in the treatment of established Rhus dermatitis either in guinea pigs or in human subjects.

2. Studies in human subjects indicate that the juice of these plants and isolated enzymes therefrom completely inactivate the toxic principles of poison ivy *in vitro*. Their activity *in vivo* is limited to a similar extracellular oxidation, limiting toxicity if applied immediately.

3. Data obtained in this investigation support the conclusion that the action of jewelweed preparations and its enzymes in detoxifying poison ivy extracts represents an oxidation of ivy toxins. This activity is demonstrable only under optimal conditions not to be anticipated in clinical poison ivy dermatitis.

4. Results obtained with jewelweed preparations do not bear out the claims previously reported for this material in treating ivy dermatitis. They do not indicate any advantage for this material over oxidants more readily available, and reported (26) to have similar activity.

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The Synthesis and Pharmacological Study of Some Mercurated Allyl Derivatives of Barbituric Acid*,†

By MORESHWAR V. NADKARNI‡ and JAMES W. JONES§

Five mercurated allyl derivatives of barbituric acid were studied in an attempt to add to the already existing knowledge about the relationship between chemical structure of mercurial diuretics and their therapeutic efficacy. Two isomeric mercurated monoallyl, two isomeric mercurated diallyl, and one mercurated triallyl derivative were prepared and their properties described. A study of their toxicity and diuretic qualities was made on rats and mice.

DIURESIS, as a result of mercurial administration, was noted by Jendrassik (1) in the latter part of the nineteenth century. The earliest compounds used for this purpose were of the inorganic type, calomel being the most frequently employed for dropsical conditions as an aid in ridding the organism of water. Inorganic mercurial compounds, however, suffer a significant disadvantage in that they tend to produce untoward side effects, chief among these being violent catharsis produced by calomel and the high toxicity of mercuric chloride. The toxicity of mercurial compounds was shown to be proportional to the amount of mercury ions present and this consideration led to the search for and development of less ionizable and, therefore, less toxic mercurials. Such compounds are the organic mercurial derivatives which ionize in solution at a slow rate so that the mercury ions are present in just sufficient concentration to produce an irritative stimulating effect on the kidney function, but not sufficient to produce untoward toxic effects. Blumenthal and Oppenheim (2), in 1913, studied several organic mercurial compounds and showed that all of these possessed diuretic properties. Later, Saxl and Heilig (3), Brunn (4), and Gunsberg (5) introduced Novasurol and Salyrgan, both of which are mercurated derivatives of organic compounds containing an allyl group. Issekutz and Vegh (6) made an important contribution by reporting a systematic comparative study of several organic mercurial compounds. Although certain attempts have been made in recent years at a quantitative comparison of diuretics, the evidence collected so far and its interpretation by various workers is conflicting. Sollmann and Schreiber (7) classified mercurial compounds into the "diuretic" and the "nondiuretic" type and showed that it required 1500 times more of "organic" than "inorganic" mercury to produce the same diuretic effect. This led to a conclusion that the diuretic effect was the result of ionized mercury. Fourneau and Melville (8) also confirmed these findings, in essence. Hunt, Walter, and Fosbinder (9) postulated that "diuretic type" mercurials possess a common structure in that they consist of a cyclic nucleus containing a solubilizing group and a mercurated side chain joined to the nucleus through an amide linkage. However, exceptions had been cited in literature where the mercurials did not strictly conform to these stipulations and still showed pronounced diuretic activity (10–12).

Thus, although a large number of mercury compounds have been prepared and investigated for their chemotherapeutic value, there exists considerable diversity of opinion among different workers in the interpretation of the relationship between physiological activity and chemical constitution of these compounds. In view of this, the question as to what extent changes in chemical union of the metal in an organic compound influence its pharmacological activity or what particular moiety or moieties are essential for this activity, must still be considered open.

An excellent opportunity to examine this question was offered by some of the barbituric acid derivatives containing an unsaturated alkyl chain, which could be readily mercurated to form compounds having possible diuretic properties. Barbituric acid molecules contain four replaceable hydrogen atoms, two of which are attached to a carbon atom forming a methylene group of the malonic acid unit, while the other two are attached to the two nitrogen atoms of the urea unit, forming a part of the cyclic amide linkage. All of these hydrogen atoms are replaceable by an unsaturated alkyl group, and it is obvious from the structure that the product obtained by mercuration of the allyl group attached to a nitrogen atom is structurally different from

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the mercurated product obtained when the allyl group is attached to the methylene carbon atom.

It was of interest, therefore, to prepare the two types of compounds and investigate pharmacologically whether the two types differed significantly in their physiological effect. Furthermore, it is a recognized fact that barbituric acid derivatives, which are extensively used as sedatives and hypnotics, produce a definite antidiuretic effect (13–17). De Bodo and Prescott (18) pointed out a direct relation between the hypnotic effect and antidiuresis produced by phenobarbital sodium in dogs.

The object of this investigation was: (*a*) to prepare the two isomeric mercurated monoallyl derivatives, two isomeric mercurated diallyl derivatives, and one mercurated triallyl derivative of barbituric acid; (*b*) to determine the acute toxicities and diuretic activities of these compounds; and (*c*), to note the effect of the increasing percentage of mercury in the molecule and of altering the position and type of attachment of the mercurated side chain upon these pharmacologic properties.

EXPERIMENTAL

Preparation of 5-Allylbarbituric Acid.—This compound was prepared by the general method described by Johnson and Hill (19). Seven and two-tenths grams of metallic sodium was dissolved in 250 cc. of absolute alcohol in a dry, 500-cc. flask, fitted with a reflux condenser having a calcium chloride tube in the upper end, and a mercury sealed stirrer. Nine grams of urea, previously dried at 100°, was then added. The mixture was warmed and stirred until solution was complete, then 30 Gm. of allylmalonic ester (20) was added. The mixture thickened to a semisolid in a short time. It was refluxed at 105–110°, with continuous stirring, for ten hours. This resulted in a pinkish semisolid. The alcohol was then removed by distillation and the residue dissolved in a minimum amount of hot water. The solution was acidified with dilute hydrochloric acid. Upon storage in a cold place for several hours, a flocculent precipitate formed. The solid was filtered off, washed with ice water and recrystallized from hot water. Yield: 11.5 Gm. or 37% of the theoretical. Melting point: 167°, corresponding to that recorded.

Preparation of 3-Allylbarbituric Acid.—Two and three-tenths grams of metallic sodium was dissolved in 50 cc. of absolute alcohol under a reflux condenser fitted with a calcium chloride tube. Sixteen grams of ethyl malonate was added, followed by 10 Gm. of allylurea, suspended in 50 cc. of absolute alcohol. The mixture was refluxed at 105–110° for twenty-four hours. In about three hours, a white precipitate formed and gradually increased in amount. The alcohol was evaporated off and the residue dissolved in a minimum of cold water. This solution was acidified with dilute hydrochloric acid, giving rise to a voluminous, orange-yellow precipitate. The solid was filtered off, washed with ice water, and purified by dissolving it in dilute sodium hydroxide and reprecipitating with dilute hydrochloric acid. The pure compound appeared as white, crystalline flakes. Yield: 7.5 Gm. or 44.7% of the theoretical. Melting point: 69°. Nitrogen: calcd., 16.67%. Found, 16.73%.

Preparation of 5,5-Diallylbarbituric Acid.—Seven and two-tenths grams of metallic sodium was dissolved in 100 cc. of absolute alcohol in a three-necked flask, fitted with a reflux condenser having a calcium chloride tube in the upper end. Four and eight-tenths grams of dry urea was added, followed by 20 Gm. of diallyl malonic ester. The mixture was refluxed at 100–105° for thirteen hours. The alcohol was then evaporated off and the residue dissolved in a minimum amount of water. Neutralization of this solution with dilute sulfuric acid gave a precipitate of 5,5-diallylbarbituric acid. The precipitate was filtered off, washed with ice water, and dried. Yield: 10.5 Gm. or 54.7% of the theoretical. Melting point: 171°, corresponding to that recorded.

Preparation of 3,5-Diallylbarbituric Acid.—Seven and four-tenths grams of metallic sodium was dissolved in 100 cc. of absolute alcohol in an apparatus as previously described. Sixteen grams of allyl malonic ester and 8 Gm. of allylurea were added and the mixture refluxed for fourteen hours. The alcohol was then evaporated off and the residue dissolved in a small amount of water. Addition of dilute sulfuric acid to the solution gave a white, flaky precipitate of 3,5-diallylbarbituric acid. This was filtered off and purified by acidifying the sodium salt solution. Yield: 4.0 Gm. or 24% of the theoretical. Melting point: 68–69°. Nitrogen: theoretical, 13.46%. Found, 13.49%.

Preparation of 3,5,5-Triallylbarbituric Acid.—Seven and four-tenths grams of sodium was dissolved in 120 cc. of absolute alcohol in a flask as described above. Eight grams of allylurea was added, followed by 20 Gm. of diallyl malonic ester. The mixture was refluxed at 100–105° for six hours, during which time a yellowish brown magma formed. The alcohol was evaporated off and the residue dissolved in water. Acidification of the solution with concentrated hydrochloric acid produced an oily material which, on standing in a refrigerator for several hours, formed a semisolid. The compound was precipitated by diluting its alcoholic solution with water and allowing the mixture to cool. Crystallization from a ligroin-ether mixture (1:1) was also used to purify the compound. Yield: 1.4 Gm. or 28% of the theoretical. Melting point: 69°, corresponding to that recorded (21).

Mercuration of the Allyl Derivatives of Barbituric Acid.—In view of the straightforward nature of the reaction between mercuric acetate and olefinic compounds, the method for mercuration of the various allyl-substituted derivatives of barbituric acid differed very little except in minor details with respect to quantities of starting materials or the time of refluxing. The general procedure was as follows: Five-hundredths mole of the mono, di-, or triallyl barbituric acid was dissolved in a suitable amount of dry methanol. In a separate flask, slightly more (about 5%) than one, two, or three equivalents of mercuric acetate, depending upon the number of allyl groups to be mercurated, was dissolved in a minimum amount of dry methanol containing four

drops of glacial acetic acid. The boiling barbiturate solution was added slowly and with constant stirring to the mercuric acetate solution. The mixture was then boiled under a reflux condenser for about twenty minutes. A heavy, amorphous precipitate of the β-methoxy-γ-acetoxymercuripropyl derivative of the barbiturate settled out on letting the mixture stand for twenty-four to forty-eight hours except in the case of the mercurated derivative of 3,5,5-triallyl barbituric acid. The latter compound remained in solution. The precipitate was filtered off and washed with methanol. In the case of mercurated 3,5,5-triallyl barbituric acid, the clear solution was permitted to stand thirty-six hours after the reaction period, then distilled under reduced pressure to remove the solvent and acetic acid. A thick, plastic material remained. This was washed with a mixture of two parts methanol and one part of water and crystallized either by allowing it to stand at room temperature for an extended time or by stirring it with an ether-ligroin mixture (5:2 by volume).

General Properties of the β-Methoxy-γ-acetoxymercuripropyl Derivatives of Barbituric Acid.—These compounds usually were white, amorphous solids, odorless when freshly prepared and washed with methanol. They developed an acetic acid odor on standing in the air due to spontaneous hydrolysis to the hydroxymercuri compounds and acetic acid. They were soluble and stable in alkaline solution and did not ionize in concentrations sufficient to form yellow mercuric oxide. They were slightly soluble in ethanol, chloroform, and acetone, but insoluble in ether, benzene, or other hydrocarbons. Upon boiling or stirring with water, the compounds were easily converted to the insoluble hydroxymercuri derivatives with the liberation of acetic acid. None of the compounds possessed a sharp or constant melting point, probably due to the slow change in composition by spontaneous hydrolysis. The yields and physical constants for the acetoxymercuri compounds were not recorded since pure products were not obtainable.

Hydrolysis of the β-Methoxy-γ-acetoxymercuripropyl Derivatives of Barbituric Acid.—Five grams of mono, di-, or tri-β-methoxy-γ-acetoxymercuripropyl derivative was added to about 150 cc. of distilled water, previously warmed to 70°, and stirred continuously for two hours without further warming. The mixture was then stirred occasionally during thirty-six hours. The resulting precipitate was then filtered off, washed several times with water, and dried in a desiccator. The yields, melting points, and analyses of the five β-methoxy-γ-hydroxymercuripropyl derivatives of barbituric acid are shown in Table I. The yields are based on the corresponding allyl-substituted barbituric acids used as starting materials.

These compounds were white or slightly grayish, amorphous solids, odorless and stable in air at room temperature. They were slightly soluble in ethanol, acetone, and polyhydric alcohols. They were soluble in one equivalent of sodium or potassium hydroxide, but insoluble in cold or hot water, ether, benzene, and other hydrocarbon solvents. The compounds having the mercurated side chain attached to nitrogen were more readily soluble in alkali than those having the side chain attached to carbon.

TABLE I.—YIELDS, MELTING POINTS, AND ANALYSES OF MERCURATED ALLYL DERIVATIVES OF BARBITURIC ACID

Compound	Yield, %	M. P., °C.[a]	Hg Analysis Calcd.	Hg Analysis Found
5-X[b] Barbituric acid	79.3	230	48.15	48.27
3-X Barbituric acid	63.4	208	48.15	48.32
5,5-Di-X Barbituric acid	65.2	295	56.89	57.01
3,5-Di-X Barbituric acid	57.3	265	56.89	56.79
3,5,5-Tri-X Barbituric acid	61.0	198	60.57	60.59

[a] With decomposition.
[b] X = β-methoxy-γ-hydroxypropyl.

PHARMACOLOGIC STUDY

Since the primary purpose of this investigation was to study the correlation between chemical structure and pharmacologic activity, it was necessary to determine the acute toxicities and diuretic activities of the mercurated compounds described above.

Acute Toxicity Study.—In order to compute the LD_{50} of the compounds, the method of probit transformation (22, 23) was applied to the percentage mortality data by observing the number of deaths produced in groups of animals after administration of graduated doses of the drug. The test solutions were prepared by dissolving the mercurial in an equivalent of sodium hydroxide solution. The dilution was adjusted to a concentration of mercury such that the amount of solution required to be injected on a weight basis did not exceed 1.2 cc. in the case of mice or 7.5 cc. in the case of rats. The *p*H of the solutions was between 7.5 and 9.0. The solutions were administered intraperitoneally. The solutions were administered in graduated increasing doses on a weight basis to groups of 10 healthy, albino rats of mixed sex, weighing 140–250 Gm. so that the largest dose resulted in a mortality greater than 50% of a group and the smallest dose resulted in less than 50% mortality. An intermediate dose was used as a check for corroboration. Using the relative percentage mortality and survivals in each case at the end of twenty-four hours after administration of a single dose, the normal deviate and the probits were calculated from statistical tables (23). The probits were plotted against the dose and the LD_{50}, which corresponded to the dose when the probit equaled 5, was read off the plot. A comparative study of the toxicities on albino rats and mice was made only in the case of the isomeric, mercurated monoallyl barbituric acids. The mice weighed from 25 to 40 Gm. and were used in groups of 20. The results of this study are shown in Figs. 1 and 2 and in Table II.

TABLE II.—ESTIMATED LD_{50} VALUES FOR MERCURATED ALLYL DERIVATIVES OF BARBITURIC ACID

Compound	LD_{50} in Mg. Hg/Kg. Rats	Mice
5-X[a] Barbituric acid	19.5	36.0
3-X Barbituric acid	8.0	17.5
5,5-Di-X Barbituric acid	30.5	..
3,5-Di-X Barbituric acid	13.5	..
3,5,5-Tri-X Barbituric acid	22.5	..

[a] X = β-methoxy-γ-hydroxymercuripropyl.

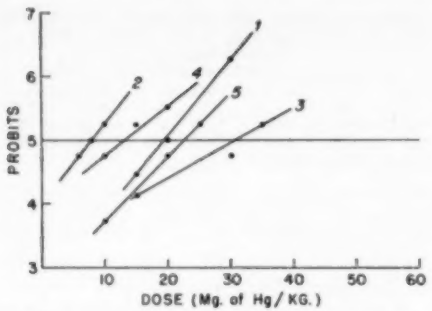

Fig. 1.—LD_{50} determination of mercurated allyl barbituric acids. Mercurated derivatives of *1:* 5-allyl barbituric acid; *2:* 3-allyl barbituric acid; *3:* 5,5-diallyl barbituric acid; *4:* 3,5-diallyl barbituric acid; *5:* 3,5,5-triallyl barbituric acid.

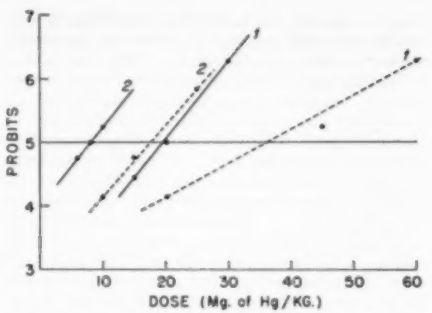

Fig. 2.—Comparative LD_{50} determination of isomeric mercurated monoallyl barbituric acids. Mercurated derivatives of *1:* 5-allyl barbituric acid; *2:* 3-allyl barbituric acid. (—— rats, - - - - mice).

In comparison to the above LD_{50} values, Long and Farah (24) reported that the intravenous LD_{50} of Mersalyl on rats is about 40.1 mg. Hg/Kg.

Diuretic Study.—The method described by Lipschitz, Hadidian, and Kerpesar (25) was followed with certain modifications. Solutions of the compounds to be tested were made as described in the acute toxicity study. The concentration of the solutions was adjusted to a mercury content such that the maximum amount to be injected on a weight basis was 1.8 cc. The *p*H of all solutions was adjusted to between 7.5 and 9.0. Healthy albino rats of mixed sex and weighing 140–270 Gm. were fasted for twelve hours prior to being used. All animals were given 25 cc./Kg. of physiological saline solution by stomach tube and divided into groups of four. One group was used as a control and received no drug. The remaining groups were injected intraperitoneally with varying doses of the test compounds. A total of 25 groups was used. One group in each series was administered Salyrgan solution equivalent to 8 mg. Hg/Kg. for comparative purposes. The volume of urine excreted by each group was noted at regular intervals up to twenty-four hours. The volumes excreted during these intervals were expressed as a percentage of the twenty-four hour totals. Excretion at the dosage level which caused maximum diuretic response was plotted against time to show the rate of excretion at the optimal diuretic dose of each compound. These results are shown in Fig. 3.

In order to calculate the diuretic activity, the volume of urine excreted during the twenty-four-hour period following administration of the mercurial was expressed as a per cent of the total amount of saline administered orally to the groups. This

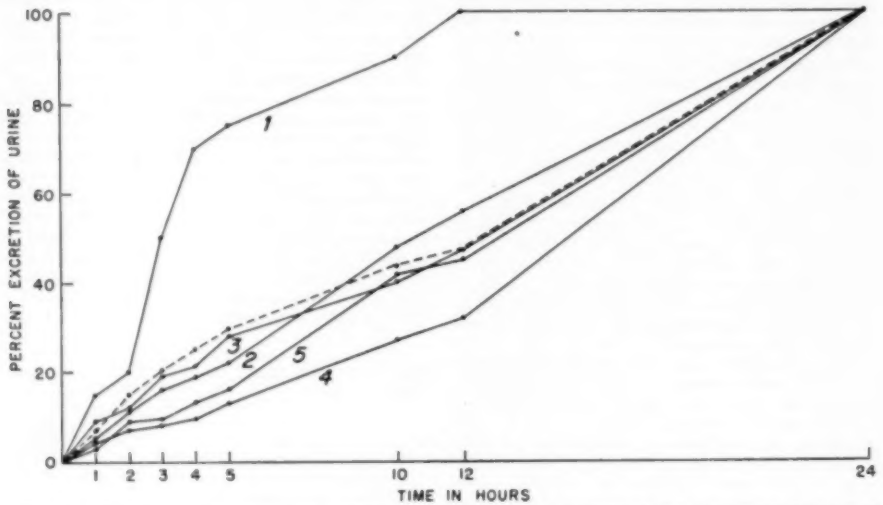

Fig. 3.—Hourly diuretic response at optimal diuretic dosages of mercurated allyl barbituric acids and of Salyrgan. Mercurated derivatives of *1:* 5-allyl barbituric acid; *2:* 3-allyl barbituric acid; *3:* 5,5-diallyl barbituric acid; *4:* 3,5-diallyl barbituric acid; *5:* 3,5,5-triallyl barbituric acid (- - - - Salyrgan).

TABLE III.—DIURETIC ACTIVITY OF MERCURATED ALLYL DERIVATIVES OF BARBITURIC ACID ON ALBINO RATS

Mercurated Barbituric Acid Derivative	Dose, Mg. Hg/Kg.	Vol. of Saline Given, Cc.	Urine Excretion 24 Hr., Cc.	Excretion, %	Excretion Control, %	Diuretic Activity	LD_{50}, Mg. Hg/Kg.	Therapeutic Index
5-Allyl	4	17.75	16.0	90.15	116.6	0.77	19.5	...
	6	15.75	5.0	31.74	116.6	0.27	..	...
	8	18.25	4.0	21.22	116.6	0.19	..	...
3-Allyl	2	15.0	18.5	123.3	82.5	1.49	8	4.0
	3	15.75	5.0	31.74	82.5	0.38	..	...
	4	17.0	1.5	9.0	82.5	0.11	..	...
5,5-Diallyl	4	20.75	13.0	62.63	133.4	0.47	30.5	...
	6	20.25	28.5	140.7	133.4	1.05	..	...
	8	19.5	26.0	133.3	133.4	1.00	..	...
3,5-Diallyl	4	16.0	37.0	231.3	139.7	1.66	13.5	3.4
	6	17.0	13.0	76.5	139.7	0.55	..	...
	8	16.75	10.0	59.7	139.7	0.43	..	...
3,5,5-Triallyl	4	17.25	36.5	211.6	139.7	1.51	22.5	5.6
	6	23.25	29.0	124.7	139.7	0.89	..	...
	8	22.25	26.5	119.1	139.7	0.85	..	...

percentage was a measure of urinary excretion independent of group weight. It has been noted that certain compounds or environmental conditions are capable of causing urine excretion greater than the total volume of liquid administered (25), which may apparently be interpreted as diuresis. True diuretic activity, independent of the influence of external factors, can be obtained by comparison with the urine excretion of the control group by the relation,

$$\text{True diuretic activity} = \frac{\%\text{ urinary excretion of test group}}{\%\text{ urinary excretion of control group}}$$

Table III shows the diuretic activities of the experimental compounds at the dosage levels used. Values greater than one denote true diuretic response. The diuretic activity of Salyrgan at the dose indicated above was found to be 1.59 and 1.66.

The therapeutic index for the compounds which exhibited true diuretic response was calculated from the relation,

$$\text{Therapeutic index} = \frac{LD_{50}\text{ in mg. Hg/Kg.}}{\text{Optimal diuretic dose}}$$

A study of the foregoing data shows that the total mercury content of the compounds had little or no bearing on their toxicity. It also shows that attachment of the mercurated side chain to an amido nitrogen rendered the compound more toxic than when the side chain was attached to the methylene carbon atom. Only those compounds which contained a mercurated side chain attached to a nitrogen atom forming part of the cyclic nucleus exhibited true diuretic response. The antidiuretic effect of a barbiturate was too strong to be neutralized by a mercurated side chain on the nitrogen atom. All the compounds studied possessed an optimum dose for diuretic response which, if exceeded, produced marked antidiuresis. The diuretic efficiency of the compounds bore no relation to the total mercury content of the molecule. Rather, it was the nature of attachment of the mercurated side chain which decided this property. In general, it may be concluded that: (*a*) definite structural requirements must be fulfilled before an organic mercurial compound will show true diuretic properties; (*b*) the antidiuretic property of a barbiturate can be annulled or it can be transformed into a diuretic compound by substituting a mercurated side chain in the proper position; and (*c*) the pharmacologic properties of these mercurated derivatives differ considerably depending upon their structural configuration.

SUMMARY

1. Two isomeric, mercurated, monoallyl derivatives, two isomeric, mercurated, diallyl derivatives, and one mercurated triallyl derivative of barbituric acid were prepared.

2. A limited pharmacologic study with respect to the toxicity and diuretic activities of these compounds was made.

3. No direct correlation between total mercury content of the compounds and the diuretic activity was evident.

4. The presence of an unsubstituted methylene group imparted an antidiuretic effect to the barbiturate. This effect could be partially or completely annulled by proper substitution with a mercurated allyl group.

5. Each compound possessed an optimum dose which produced maximum diuresis. An increase of dosage beyond this level resulted in marked antidiuretic effect.

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Note

A Note on Ferric Cacodylate Detoxification*

By E. E. MOORE and L. O. SOMMERS†

FERRIC cacodylate has been used for years in conditions for which iron and arsenic are indicated. While the product, in general, has been satisfactory, occasional lots have been too toxic for use (1–3).

The formula for ferric cacodylate is given in the National Formulary VIII as $Fe[(CH_3)_2AsO_2]_3$, which indicates 11.96 per cent Fe to 48.15 per cent As. The N. F. limits are "not less than 11 per cent and not more than 16 per cent of Fe, and not less than 41 per cent and not more than 45 per cent of As." It has been observed that the N. F. product, which contains excess Fe, gives a less toxic solution than does a product containing the theoretical percentages of Fe and As.

EXPERIMENTAL

Three solutions, each containing 15 mg. ferric cacodylate per cc., were prepared for testing. Solution *A* was made from ferric cacodylate containing the theoretical percentages of Fe and As. Solution *B* was made from ferric cacodylate containing excess Fe corresponding to N. F. standards. Solution *C* was made from the same ferric cacodylate as solution *A* and then sufficient colloidal iron (dialyzed iron, Merck) was added to give a ratio of Fe to As, the same as in solution *B*.

All three solutions had the same toxicity. When the solutions were heated in an autoclave at 15 pounds pressure for twenty minutes, colloidal iron formed. (If the solutions are overheated a precipitate will form.) These sterilized solutions were tested, and it was found that the toxicity of solution *B* was reduced much more than that of solutions *A* and *C*. (See Table I.)

Electrophoretic measurements were made on all the above-mentioned solutions. In the case of the sterilized solution of compound (*B*) the colloid migrated to the negative pole and a clear colorless liquid was left at the positive pole. Neither of the other solutions gave a clear colorless liquid at the positive pole.

TABLE I.—TOXICITY LD_{50} MG./KG. IN MICE

Solution	Before Sterilization	After Sterilization
A	250	400
B	250	800
C	250	400

Biltz (4) demonstrated that the value of iron compounds in the treatment of arsenous acid poisoning was due to adsorption of the arsenic compound by freshly precipitated ferric hydroxide. It appears probable that a similar adsorption and detoxification occurs during the sterilization of ferric cacodylate solutions. Colloidal iron oxide is formed during sterilization, as may be seen by the deepening in color of the solution, and it is only after sterilization that the detoxification may be noted. The higher the ratio of Fe to As, the more of the latter will be adsorbed. The addition of dialyzed (colloidal) iron to the solution is of no value because the iron is already in the colloidal state when added to the cacodylate; hence it cannot present a freshly precipitated surface to adsorb the arsenic compound.

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Book Reviews

Author's Guide for Preparing Manuscript and Handling Proof. John Wiley and Sons, New York, 1950. xi + 80 pp. Illustrated. 15.5 x 23.5 cm. Price $2.

The task of preparing a manuscript for publication and supervising its progress through the various phases to final appearance is a process that can be complex and expensive, or fairly simple and reasonable in cost. The author is often the controlling factor in this decision, and if he is not already familiar with publishing procedures, he would do well to read John Wiley and Sons' new book, *Author's Guide for Preparing Manuscript and Handling Proof.*

The purpose of this book is twofold: to guide the author in the efficient preparation of his material, both manuscript and illustrations; and to inform him of the publishing procedure from the time he submits his manuscript until he has the printed publication in hand.

The book itself is, of course, an ideal example of organization and form. One of the most valuable features is the excellent section on the handling of illustrative material—a field where too many authors fail, with resulting high costs and frayed author-publisher relations.

A complete glossary of printing terms, and checklists for the author on each stage of proof are also provided.

Copyright practices, another field where authors often meet complications, are handled briefly but clearly in one section of the book. A chapter stressing the importance of consistency in editorial style and one entitled "How to Proofread" are other important chapters.

Authors of articles for periodicals, as well as books, will find this a handy reference book, regardless of their subject matter, and they will also find that by heeding some of the book's suggestions, publishing expenses will be substantially reduced, and their satisfaction with the final results increased.

Quantitative Ultramicroanalysis. By PAUL L. KIRK. John Wiley and Sons, New York, 1950. vii + 310 pp. Illustrated. 14 x 22 cm. Price $5.

Many fields of research, whether of necessity or convenience, are leaning toward use of ultramicro quantities for analysis. The importance of ultramicro techniques has been increased by development of radioisotopes, by the rapid growth of interest in cell physiology, and by the development of new antibiotics and pharmaceuticals available in limited quantities. *Quantitative Ultramicroanalysis*, therefore, constitutes a valuable and timely addition to the technical library.

This book presents in a clear and precise manner the techniques and equipment for analysis of microgram quantities of materials. It describes in adequate detail the instruments used and points out their advantages and disadvantages. In almost every case the author describes more than one operation or instrument that has been used for a given purpose, but he limits the treatise to those instruments and methods which, in his opinion, have most general application. Of particular value are his comments on fragility, inherent errors, and ease of manipulation as regards the various equipment. The text covers practically all of the common quantitative manipulations, weighing, transferring liquids, titrating, extracting, but it also extends to spectrophotometry, gas analysis, and special techniques of biological research. There are sections devoted to specific determinations, these sections describing in detail the determination of metallic ions, amino nitrogen, halogens, reducing sugars, etc. Also included is a short but interesting chapter on ultramicro physical methods. The book is well documented throughout.

The reviewer believes that this book will be of interest to research laboratories of all types, but especially valuable for biochemical and pharmaceutical research groups. It is written with sufficient clarity and directness to be suitable for use by advanced students, and it is a readable and fascinating introduction to the subject of ultramicroanalysis that will be enjoyed by most chemists, regardless of their field.

Electronic Interpretations of Organic Chemistry. 2nd ed. By A. EDWARD REMICK. John Wiley and Sons, New York, 1949. vii + 600 pp. Illustrated. 15 x 23.5 cm. Price $6.

The second edition of this book retains the excellence of the first as an introduction to and survey of the electronic concepts of organic chemistry. The arrangement is essentially the same with the very useful appendix containing symbols and basic principles so listed as to be easily referred to during reading of the text. The author has added new material to each chapter and included a new section on stereochemistry, increasing the size of the book about 100 pages. The author has presented the subject material with such clarity that the book is especially suitable for students and newcomers to the field. Usually he carefully explains the problem involved before presenting experimental evidence and afterward indicates the limitations of the phenomena when used predictively. Methods of measurement have not been discussed in sufficient detail to hamper the reader or lose the train of thought.

BOOKS RECEIVED

Multi-Enzyme Systems. By MALCOLM DIXON. New York: Cambridge University Press, 1949. 100 pp. 12.5 x 19 cm. Price $1.75.

The Heterocyclic Derivatives of Phosphorus, Arsenic, Antimony, Bismuth, and Silicon. (Series: The Chemistry of Heterocyclic Compounds.) By FREDERICK GEORGE MANN. Interscience Publishers, Inc., New York, 1950. ix + 180 pp. Illustrated. 15.5 x 23.5 cm. Price: Series Subscribers—$4.20; Nonsubscribers—$5.25.

Chemical Activities of Fungi. By JACKSON W. FOSTER. Academic Press, Inc., New York, 1949. xviii + 648 pp. Illustrated. 15.5 x 23.5 cm. Price $9.50.

A Laboratory Manual for Organic Pharmaceutical Products. By CHARLES O. WILSON AND ROBERT F. DOERGE. Hemphill's, Austin, Tex., 1950. vi + 143 pp. 22.5 x 27.8 cm. Price $2.50.

Organic Chemistry. By PAUL KARRER. 4th English ed. By H. V. SIMON AND N. G. BISSET. Elsevier Publishing Co., Inc., New York, 1950. xxi + 973 pp. Illustrated. 17 x 26 cm. Price $8.50.

The Chemistry of Industrial Toxicology. By HERVEY B. ELKINS. John Wiley and Sons, Inc., New York, 1950. ix + 406 pp. Illustrated. 14 x 22 cm. Price $5.50.

Heavy Metal Prosthetic Groups and Enzyme Action. By OTTO WARBURG (translated by ALEXANDER LAWSON). Oxford University Press, New York, 1949. xii + 230 pp. Illustrated. 14 x 22.3 cm. Price $3.75.

Penicillin—Its Practical Application. 2nd ed. By SIR ALEXANDER FLEMING. C. V. Mosby Company, St. Louis, 1950. xiii + 491 pp. Illustrated. 13.5 x 22 cm. Price $7.

Selling to the Federal Government. Published by the Bureau of National Affairs, Inc., Washington, D. C., 1949. iii + 348 pp. 21 x 28 cm. Price $5.

Biologie der Goethezeit. EDITED BY ADOLF MEYER-ABICH. Waltham, Mass.: The Chronica Botanica Co., 1949. 302 pp. Illustrated. 15.5 x 23.5 cm. Price $5.50.

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Electronic Interpretations of Organic Chemistry. 2nd ed. By A. EDWARD REMICK. John Wiley and Sons, New York, 1949. vii + 600 pp. Illustrated. 15 x 23.5 cm. Price $6.

Quantitative Ultramicroanalysis. By PAUL L. KIRK. John Wiley and Sons, New York, 1950. vii + 310 pp. Illustrated. 14 x 22 cm. Price $5.

Directory of Biological Laboratories. 4th ed. Published by Burns Compiling and Research Organization, Chicago, 1949. 140 pp. 15.3 x 23 cm. Price $3.

1950 Pharmacy State Board Questions and Answers. By RALPH J. MILL. Clark and Wilson Company, Detroit, 1949. 100 pp. 21.5 x 28 cm. Price $3.

Methods in Medical Research. Volume II. JULIUS H. COMROE, JR., Editor-in-Chief. Year Book Publishers, Inc., Chicago, 1950. xvi + 361 pp. 14 x 22.5 cm. Illustrated. Price $6.50.

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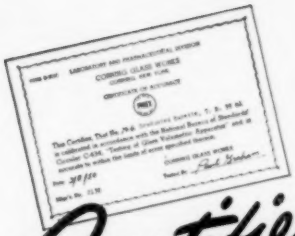

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